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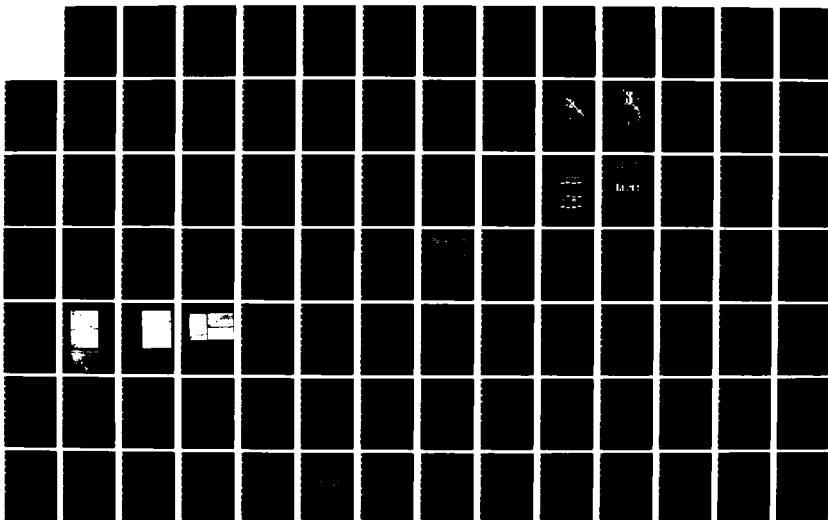
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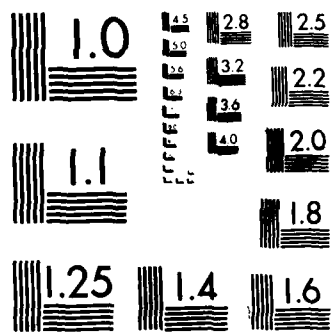
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Mechanisms of Bunyavirus Virulence: A Genetic Approach

Annual Report

Neal Nathanson, M.D.

December 1984

(03 Year)

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20. (Abstract)

→ The construction and characterization of hybridomas making monoclonal antibodies against La Crosse and Tahyna viruses is described. These antibodies have been characterized by the following tests: (1) ELISA, (2) neutralization, (3) hemagglutination inhibition (HI), (4) cross reactivity against California serogroup viruses, (5) immunoprecipitation of viral protein. These antibodies have been shown to be useful tools for the classification of new viral isolates and for the phenotyping of reassortant viruses. They have been used to select variant viruses, which can escape neutralization.

The virulence of California serogroup viruses is being studied by genetic analysis. (a) Two parent viruses, have been selected to represent a virulent prototype (La Crosse original) and an avirulent prototype (Tahyna 181/57). (b) The pathogenesis of these two viruses has been studied by infections of suckling mice, and it has been found that the neuroinvasive virulent virus replicates well in striated muscle with subsequent viremia, while the avirulent virus does not. (c) Reassortants have been constructed from these two parents and partially phenotyped. (d) Variant viruses, selected with neutralizing monoclonal antibodies, are under study for reduced virulence. ↗



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#### FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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4. Papers and manuscripts	

Gonzalez-Scarano F, Shope RE, Calisher CH, and Nathanson N. Characterization of monoclonal antibodies against the G1 and N proteins of La Crosse and Tahyna, two California serogroup bunyaviruses. *Virology*, 120:42-53, 1982.

Gonzalez-Scarano F, Shope RE, Calisher CH, and Nathanson N. Monoclonal antibodies against the G1 and nucleocapsid proteins of La Crosse and Tahyna viruses. In *California Serogroup Viruses*, edited by CH Calisher and W Thompson, pp. 145-156, Alan Liss, New York, 1983.

#### Appendix 1

Gonzalez-Scarano F, Pobjecky N, and Nathanson N. La Crosse bunyavirus can mediate pH-dependent fusion from without. *Virology*, 132:222-225, 1984.

#### Appendix 2

Janssen R, Gonzalez-Scarano F, and Nathanson N. Mechanisms of bunyavirus virulence: comparative pathogenesis of a virulent strain of La Crosse and an avirulent strain of Tahyna virus. *Laboratory Investigation* 50:447-455, 1984.

#### Appendix 3

Gonzalez-Scarano F, Janssen R, Najjar J, Pobjecky N, and Nathanson N. An avirulent G1 glycoprotein variant of La Crosse bunyavirus with a defective fusion function. *J Virology*, in press, 1985.

#### Appendix 4

Gonzalez-Scarano F. La Crosse glycoprotein undergoes a conformational change at the pH of fusion. *Virology*, 140:209-216, 1985.

#### Appendix 5

Najjar JA, Gentsch JR, Nathanson N, and Gonzalez-Scarano F. Epitope analysis of the G1 glycoprotein of La Crosse virus using variant viruses selected with monoclonal antibodies. *Virology*, in press, 1985.

#### Appendix 6

Janssen R, Nathanson N, Gentsch J, and Gonzalez-Scarano F. Polygenic determination of the virulence of reassortants between a La Crosse and Tahyna viruses. Manuscript, 1985.

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\* Please see prior Progress Reports for these reprints.

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I. Introductory Note: Scope and duration

As negotiated, the budget for this contract for the 03 year, 12-01-83 through 11-30-84, is summarized below.

Components of Budget	03 Year
Total Direct Costs	61,772
Indirect Costs (65% TDC)	36,902
Total Contract	98,674

Since it was clear that only a part of the original proposal could be funded on a contract of this size, we have selected the DEVELOPMENT, CHARACTERIZATION, AND UTILIZATION OF HYBRIDOMAS as the focus for this project. The investigators collaborating on this aspect of the program are listed below.

Neal Nathanson	Professor	Pathogenesis and immunology
Francisco Gonzalez	Assistant Professor	Hybridomas and variant viruses Fusion studies
Jon Gentsch	Assistant Professor	Genetics and protein chemistry
Robert Janssen	Postdoctoral Fellow	Pathogenesis and genetics

PREVIEW. The following presentation attempts to give an overview of the important findings from this contract through the first three years. Those findings which are the oldest are detailed in the reprints and will be summarized only briefly. Recent findings which we consider particularly significant will be described at greater length. From the perspective of this contract, we consider these to be:

- (j) Classification of epitopes on the G1 glycoprotein with monoclonal antibodies
- (f) and (g) Demonstration that La Crosse virus mediates pH-dependent fusion
- (h) and (i) Selection of variant viruses with neutralizing monoclonal antibodies.

## 2. Hybridomas against La Crosse and Tahyna viruses

### (a) Uses of hybridomas

The central theme of our studies is to use the California virus system as a model to study the factors which determine the outcome of acute viral encephalitis. Among these factors are virus determinants (neuroinvasiveness and neurovirulence) and host determinants (recovery from infection and protection against subsequent infection). We plan to use monoclonal antibodies as a tool to investigate several aspects of this model.

(i) To make reassortant viruses from virulent and avirulent parent viruses, monoclonal antibodies can be used to rapidly phenotype putative reassortants from both G1 and N proteins.

(ii) To select antigenic variants of parental virus, growth in the presence of neutralizing monoclonal antibodies will select efficiently for variants. These variant viruses can be used for two distinct purposes: (a) To determine if variants show changes in their virulence, which could occur if the G1 protein is an important determinant of virulence. Our results with one variant (see below) have now validated this hypothesis. (b) To group the monoclonones themselves according to their reactivity with a panel of variant viruses.

(iii) To identify and map biological functions of the viral glycoproteins. Important functions associated with glycoproteins of enveloped viruses are: binding to erythrocytes (hemagglutination), binding to host cells (neutralization), and fusion of membranes (hemolysis or cell fusion).

(iv) To determine the protective role of antibodies directed against antigenic determinants of California encephalitis virus glycoproteins.

In other enveloped viruses antibodies against certain sites on the glycoprotein will neutralize, but the efficiency may differ markedly, depending on whether the site is involved in attachment or in fusion. Also, there may be a synergistic effect of neutralizing antibodies against two different antigenic sites.

Antibodies which fail to neutralize may have several different effects: in some cases non-neutralizing antibodies block neutralization, while in other instances they are protective in vivo.

Finally, the properties of the antibody itself (isotype, avidity, complement fixation, ability to mediate virolysis and cytolysis, and the like) may influence its protective efficiency. Such questions can be studied with monoclonal antibodies much more precisely than could ever be accomplished with polyclonal antisera.

### (b) Immunization of mice and construction of hybridomas

La Crosse and Tahyna viruses were selected for this study because they represent antigenically distinct strains with relative differences in virulence in mice, La Crosse being the more neuroinvasive (kills after ip injection) and Tahyna the more avirulent (fails to kill after ip injection above 2 weeks of age). Also, it had been shown that reassortants could readily be made between these two viruses.

To immunize mice, advantage was taken of the fact that La Crosse and Tahyna viruses produce active infections in mice; intraperitoneal or intracerebral injection was used, to initiate a severe infection with some deaths. Survivors were given a booster injection of virus and 2-4 days later mice were sacrificed and spleen cells prepared.

Spleen cells from LAC or TAH immunized mice were fused with a BALB/c myeloma line (P3 x 63 clone 653) which is a nonsecretor. In HAT medium this line will be killed since it cannot utilize the purine salvage pathway (HPRT-); this function is provided by the lymphocyte partner in the hybrid cell. A mixture of spleen:myeloma cells at a 10:1 ratio was made and PEG 1000 used as fusing agent. The mixture was plated in micro wells,  $5 \times 10^5$  cells per well.

After 2-3 weeks of incubation, wells with visible colonies were tested for anti-viral antibody in ELISA assay, using partially purified virus as antigen. Positive cultures were cloned in 0.25% agarose and individual colonies were transferred to flasks and again tested for antibody. Hybridoma cells were maintained in 15% serum and supernates collected as a cell culture source of monoclonal antibody. For high titer preparations,  $10^7$  hybridoma cells were injected ip into Pristane-primed BALB/c mice and ascitic fluids collected 1-2 weeks later. Antibody titers of ascitic fluids were usually about 100-fold higher than titers of tissue cultures supernates. For neutralization, HI, CF, and ELISA, the ascitic fluid served well, but tissue culture supernate or purified immunoglobulin was required for clean immunoprecipitations. Cells are stored in a serum-DMSO mixture in liquid N<sub>2</sub>.

(c) Characterization of monoclonal antibodies

To characterize the LAC and TAH hybridomas, each monoclonal was used to immunoprecipitate virus proteins from an S35 amino acid labelled lysate of infected cells, and each was typed as to immunoglobulin class by an RIA. In addition, each was tested in ELISA, neutralization (N), and hemagglutination (HI) systems, against each of 11 California serogroup viruses. The essential results are set forth in TABLE I and may be summarized (see papers for further detail):

(i) Of 23 monoclonal, 15 were directed against the G1 glycoprotein and 8 against the N (nucleocapsid) protein, while none were against the G2 glycoprotein or the L polypeptide.

(ii) Of the 15 G1 clones, 11 both neutralized and had HI activity, one had HI activity only, and 3 were neutralization and HI negative. From this it was inferred that the G1 glycoprotein had at least two antigenic sites. One site was postulated to bind to receptors on both erythrocytes and BHK cells, accounting for the concordance of neutralization and HI results. The other site appeared uninvolved in attachment to receptors. More recent epitope studies (see below) suggest that there is only one antigenic site for both groups of antibodies.

(iii) Of the 15 G1 clones 4 were type-specific, ie, reacted with the immunizing virus only, 3 were almost type-specific, while 8 were cross-reactive.

(iv) None of the 8 nucleocapsid clones showed neutralizing or HI activity, as expected.

(v) Of the 8 nucleocapsid clones, only one was type-specific, while 7 were cross-reactive.

(vi) The 15 G1 clones were isotypized as IgG1 (7 clones), IgG2a (6 clones), or IgG2b (2 clones). By contrast, the 8 nucleocapsid clones were IgM (5 clones), Ig2a (2 clones) or undetermined (1 clone).

TABLE I  
Characterization of 23 monoclonal antibodies against La Crosse (LAC)  
and Tahyna (TAH) viruses

Clone No.	Immu- nizing Virus	Protein Precipi- tated	Ig Class	Type-Specific  Cross-Reactive	<u>Serologic Test</u>			
					ELISA	NT	HI	FI
807-09	LAC	GI	IgG2a	S	+	+	+	+/-
807-15	LAC	GI	IgG2b	S	+	+	+	+
807-18	LAC	GI	IgG1	S	+	+	+	+
807-35	LAC	GI	IgG1	S	+	+	+	+
807-31	LAC	GI	IgG1	S	+	+	+	+
807-12	LAC	GI	IgG2a	C	+	+	+	+
807-22	LAC	GI	IgG2a	C	+	+	+	+
807-33	LAC	GI	IgG2a	C	+	+	+	+
807-25	LAC	GI	IgG2b	S	+	+	?	+
807-26	LAC	GI	IgG2a	S	+	-	-	-
807-21	LAC	GI	IgG2a	C	+	-	-	-
807-13	TAH	GI	IgG1	C	+	+	+	?
813-48	TAH	GI	IgG1	C	+	+	+	-
813-77	TAH	GI	IgG1	C	+	+	+	ND
814-443	TAH	GI	IgG1	C	+	-	+	-
820-374	LAC	N	IgM	C	+	-	+	-
807-28	LAC	N	IgG2a	C	+	-	-	ND
807-32	LAC	N	IgM	C	+	-	-	ND
807-13	LAC	N	IgM	C	+	-	-	-
807-02	TAH	N	IgG2a	S	+	-	-	ND
814-08	TAH	N	?	C	+	-	-	ND
814-48	TAH	N	IgM	C	+	-	-	ND
814-87	TAH	N	IgM	C	+	-	-	ND

N: nucleocapsid. NT: neutralization test. HI: hemagglutination inhibition test. FI: fusion inhibition. S: type-specific. C: cross-reactive. Fusion inhibition: a 1:50 dilution of ascitic fluid gives a fusion index of 0.70 or lower, compared to a control index of 0.90 or higher. Antibody 807-25 was originally classified as non-neutralizing but later ascitic fluids were clearly neutralizing. Antibody 807-13 was originally neutralizing but later ascitic fluids show minimal neutralizing activity.

(d) Additional anti-G1 hybridomas

To insure a more complete delineation of the antigenic sites on the important G1 glycoprotein, additional hybridomas have been made to enlarge our basic panel. These are summarized in TABLE 2.

(e) Attempts to construct anti-G2 hybridomas

The function(s) of the LAC G2 glycoprotein is currently unknown. Since low pH mediated fusion has recently been described for LAC virus (Gonzalez-Scarano et al, 1984) it would be of interest to examine the effect of anti-G2 antibodies on this function. In addition we would like to examine the possible role of G2 antibodies in in vivo protection and virus neutralization. Past efforts to construct hybridomas against the G2 glycoprotein have utilized a protocol in which mice were hyperimmunized with La Crosse virus and after fusion by our standard protocol hybridoma cultures were screened for anti-G2 antibodies using a partially purified G2 preparation as an ELISA antigen. As detailed in our previous Progress Report, we succeeded only in obtaining additional anti-G1 specific hybridomas (TABLE 2).

More recently, Dr. Gentsch has used large amounts of low pH (pH 5.4) treated, UV inactivated La Crosse virus as an immunogen. Since low pH mediated fusion of BHK-21 cells (Gonzalez-Scarano et al, 1984) has been demonstrated for La Crosse virus, we hoped that such treatments of LAC virus would expose new antigenic sites on the G1 and/or G2 glycoproteins. We have isolated an additional 6-8 monoclonal antibodies against acid-treated virions, but unfortunately all are against the G1 glycoprotein. It is interesting that some of these antibodies recognize acid specific antigenic determinants of the G1 protein (Najjar et al, 1985). It is therefore obvious from our monoclonal antibody studies and those of Laura Kingsford and Leo Grady that G2 specific La Crosse monoclonal antibodies are rare, and in fact none have yet been isolated.

Our current efforts exploit the observation (Gonzalez-Scarano, 1985) that when intact virions are digested with certain proteolytic enzymes, the G1 protein is cleaved while G2 remains intact. Pronase is currently being used for this purpose, since it appears to totally digest G1 while leaving G2 unaltered, to the extent that can be judged from 12.5% SDS-PAGE. Following fusion of splenocytes from mice immunized with pronase-treated virions, hybridoma supernates are screened with the same antigen.

Currently, 10 hybridomas have been selected using this protocol, and the supernates are now being tested by immunoprecipitation. Other mice that have been immunized with the digested virus are also available, should additional fusions prove necessary.

(f) Demonstration that La Crosse virus mediates pH-dependent fusion

Glycoproteins of enveloped viruses mediate several important biological functions. These are: (i) attachment to cellular receptors; (ii) attachment to erythrocytes; (iii) fusion of viral envelope to cellular membranes; and (iv) neuraminidase or other enzymatic activity. Of the functions listed above, the putative fusion function of bunyavirus glycoproteins had never been demonstrated.

Over the past few years, many enveloped viruses have been shown to have lipid binding and membrane fusing properties which become manifest only upon exposure of the virions to acidic pH. These properties may be necessary in order for virions to extrude their nucleocapsids into the cellular cytoplasm following uptake by endocytosis into acidic polysomal vacuoles or lysosomes which have been called receptosomes or endosomes.

TABLE 2

Additional 21 monoclonal antibodies against the GI protein LAC and TAH viruses  
data of F. Gonzalez-Scarano, J. Gentsch, and N. Pobjecky, 1985

Clone No.	Immunizing Virus	Protein Precipitated	Serological Test			
			ELISA	NT	HI	FI
807-05	LAC	GI	+	+	+	-
807-07	LAC	GI	+	-	*	ND
807-17	LAC	GI	+	-	*	-
813-57	TAH	GI	+	-	*	-
813-71	TAH	GI	+	-	*	-
813-72	TAH	GI	+	-	*	ND
814-91	TAH	*GI	+	-	*	-
820-260	LAC	GI	+	+	*	+
900-03	LAC	GI	+	+	*	ND
900-27	LAC	GI	+	+		+/-
900-62	LAC	GI	+	+		+/-
900-04	LAC	GI	+	-	*	ND
900-05	LAC	GI	+	-	*	ND
900-08	LAC	GI	+	-	*	ND
900-11	LAC	GI	+	-	*	ND
900-13	LAC	GI	+	-	*	ND
900-19	LAC	GI	+	-	*	ND
900-25	LAC	GI	+	-		-
900-29	LAC	GI	+	-		-
900-61	LAC	GI	+	-		-
900-69	LAC	GI	+	-		-

\* HI: not yet run. Isotype and cross-reactivity to be determined. NT: neutralization test. FI: fusion inhibition.

Although fusion of virion envelopes with cellular membranes can be visualized with the electron microscope, routine demonstration of fusion by this method is difficult. Therefore, a variety of indirect measures of fusion have been introduced to characterize the parameters of this glycoprotein function. Among these, low pH mediated hemolysis of red blood cells, and cell-to-cell fusion of tissue culture cells are the simplest. Cell-to-cell fusion may employ virus absorbed on cells (fusion from without or FFWO) or viral glycoproteins expressed on the surface of infected cells (fusion from within or FFWI).

Cell fusion (FFWO) was demonstrated by adaptation of the method of White and colleagues (Gonzalez-Scarano et al, 1984). Sucrose gradient-purified La Crosse virus was added to uninfected BHK cells and maintained at 4C for one hour, following which the inoculum was removed. The cells were briefly (30-60 seconds) exposed to pre-warmed MEM with 0.2% BSA containing 10 mM Hepes and 10 mM Morpholinopropanesulfonic acid (MOPS) or Morpholinoethanesulfonic acid (MES) buffer adjusted to the appropriate pH with NaOH. The buffer was removed and the cells incubated at 37C under neutral pH for 30-60 minutes, after which the monolayer was fixed and stained.

The fusion index equals  $(I-N/C)$  where N is the number of nuclei and C is the number of cells. The index was determined by directly counting representative fields. At 37C and 30 minutes, maximum fusion was obtained at pH 5.0-6.0. The amount of virus used in each experiment was a critical determinant of the extent of fusion. The temperature at which the cells were incubated following exposure to low pH was important also. At low temperatures, cell fusion does not proceed at all, and it is slow at 31.5C. Fusion also occurs with virus that has been inactivated by exposure to ultraviolet light to reduce the PFU titer by 100,000-fold.

Fusion from within (FFWI). Fusion has now been demonstrated to occur as a consequence of infection (FFWI), by N. Pobjecky and coworkers in our laboratory. BHK-21 cells are infected with an moi of 1.0, and incubated 16 hours at 35C. The infected monolayer is washed with PBS and incubated with MOPS buffer, for 30 minutes, at 37C. At pH between 5.0 and 6.0 dramatic fusion occurs (index of 0.8 or greater).

In collaboration with Dr. Jonathan Smith, USAMRIID, we have looked at FFWI in the electron microscope. The images provide a vivid demonstration of the "melting" away of plasma membranes during the fusion process. At high magnification virions can be seen between apposed plasma membranes, and it is unclear whether these extracellular virions are responsible for "FFWI". Alternatively, G1 and G2 synthesized within infected cells and transported to the plasma membrane, could be capable of mediating FFWI.

By analogy with other systems, the demonstration that LAC has a pH dependent fusion function extends the putative entry pathway involving acidic vesicles to another family of enveloped viruses.

Blocking of fusion with monoclonal antibodies. The FFWI system has made it possible to determine whether anti-G1 monoclonal antibodies are capable of blocking the fusion function, after virus has attached to the cell surface. BHK-21 monolayers, 16 hours after infection, are washed and overlaid with dilutions of antibody. After 30 minutes incubation at pH7, MOPS buffer is added at pH 5.5 and 37C, and the index determined 30 minutes later. Antibodies are capable of blocking fusion, and there appears to be a general correlation between anti-fusion activity and neutralization titer (TABLE 3).



TABLE 3

Correlation of anti-fusion activity of monoclonal antibodies and neutralization titer for anti-GI protein antibodies\*

Antibody Number	Neutra- lization Activity	HI Activity	Epitope Group	Log <sub>10</sub> Neutralization Titer*	Fusion Inhibition Activity
807-31	+	+	1	4.6	3+
807-25	+	+	4	4.6	3+
807-33	+	+	3	4.0	2+
807-260	+	ND	2	4.0	2+
807-12	+	+	3	4.0	2+
807-35	+	+	1	3.7	3+
807-18	+	+	1	3.4	2+
807-09	+	+	1	2.8	+/-
807-22	+	+	5	2.8	2+
807-15	+	+	1	2.8	1+
900-27	+	ND	ND	2.5	+/-
900-62	+	ND	ND	2.5	+/-
813-48	+	+	4.5	2.2	+/-
813-71*	+?	ND	1	1.6	-
807-05	-	ND	ND	1.3	-
807-13*	-?	-	ND	1.3	-
807-17	-	ND	4.5	1.3	-
807-21	-	-	4	1.3	-
807-26	-	-	4	1.3	-
813-57	-	-	ND	1.3	-
814-443	-	ND	4.5	1.3	-
814-91	-	+	ND	1.3	-
900-25	-	ND	ND	1.3	-
900-29	-	ND	ND	1.3	-
900-61	-	ND	ND	1.3	-
900-69	-	ND	ND	1.3	-

\* Neutralization titer and fusion inhibition activity on same ascitic fluids. FI activity: - index 0.9; +/-, index 0.9; +, index 0.7-0.9; ++, index 0.4-0.7; +++ index 0.4. Antibodies needing a check: 813-71 borderline neutralization; 807-13 previously a neutralizing antibody. FI: data of N. Pobjecky, 1985.

(g) Conformational alteration in the G1 glycoprotein at reduced pH

The activation of fusion function in the orthomyxoviridae is associated with a conformational change of the hemagglutinin molecule which occurs upon exposure of the virions to an acid environment. A similar phenomenon can be demonstrated with La Crosse G1 glycoprotein (Gonzalez-Scarano, 1985). Tryptic cleavage of purified virions at 23°C results in cleavage of G1 into a major peptide of about 95Kd (and no cleavage of either N or G2), and fragments of low molecular weight that are not resolved in a 12.5% acrylamide gel. If prior to protease treatment the virions are exposed to an acidic environment, the peptide pattern is different, and the G1 is now cleaved into a 82Kd fragment as well as peptides in the 10-15Kd range. The pH range that results in different cleavage patterns correlates with the pH range that leads to activation of the fusion function.

Alteration of the cleavage pattern following exposure of the virus to an acid environment, can be demonstrated with a variety of proteases, including chymotrypsin, fibroinolytin, elastase, bromelain, and pronase. The minor glycoprotein, G2, is not as susceptible to protease cleavage as G1. Following exposure to acid (pH 5.8), G2 is partially cleaved by fibroinolytin. In a series of timed reactions, this cleavage is seen to occur only after G1 has been extensively degraded, suggesting that the major glycoprotein may protect G2.

Additional evidence of the presence of a conformational change in the G1 glycoprotein at the pH of activation of the fusion function comes from studies with monoclonal antibodies. As part of the characterization of epitopes, monoclonal antibodies against the G1 protein were tested in an ELISA assay with virus that had been treated with acid. Binding studies are illustrated in the attached reprint (Gonzalez-Scarano, 1985). Antibody 807-17 demonstrates reduced binding to virus that has been acid-treated. Antibody 807-18, also illustrated, shows the more typical pattern, that is no difference in the binding of virus that has been exposed to acid. Control ascites fluids consisted of antibodies to the nucleocapsid protein.

G1 (120 kilodaltons) and G2 (34 kilodaltons) are present in approximately equimolar amounts in the virion envelope. Either molecule, or both, could be involved in FFWO. G1 undergoes a conformational change at acidic pH altering both tryptic cleavage and antigenic sites but, in the absence of sequence information, it is not possible to predict whether it, or G2, contains the fusion peptide.

(h) Variant viruses selected with neutralizing monoclonal antibodies specific for the G1 glycoprotein

One established application of monoclonal antibodies is the selection of variant viruses. Such variants may then be used for: (i) grouping of epitopes into antigenic sites; (ii) for the mapping of sequential epitopes onto the amino acid sequence of the G1 glycoprotein; and (iii) the use of variants to delineate submolecular determinants of virus virulence.

F. Gonzalez has selected a series of 35 variants using 11 neutralizing monoclonal antibodies, according to standard methods (Gonzalez-Scarano et al, 1983). These variants are summarized in TABLE 4. In addition, he has selected some "double" variants, by sequential passage of parent virus in the presence of two different antibodies (TABLE 5).

TABLE 4

Neutralizing anti-G1 protein monoclonal antibodies and the frequency and virulence of antigenic variants selected by them\*

Epitope	Immunizing Antibody	Virus	Specific (S) or Cross-Reactive (C)	Frequency of Variants (log <sub>10</sub> )	Virulence
1	807-31	LAC	S	-6.0	+
	807-09	LAC	S	-3.7	+
	807-35	LAC	S	-5.3	+
	807-13	TAH	C	-5.9	+
	807-18	LAC	S	-5.3	+
	807-15	LAC	S	-6.2	+
2	829-60	LAC	ND	-5.4	+
3	807-12	LAC	C	-5.0	+
	807-33	LAC	C	-4.1	R
4	807-25	LAC	ND	-6.4	+
5	807-22	LAC	C	-6.3	R

\* Characteristics of these antibodies have been published (Gonzalez-Scarano et al, 1982; 1983). Specificity was determined in neutralization tests with a panel of California serogroup viruses. Selection of variants is described in the methods section. Virulence was based on subcutaneous injection of suckling mice with 1000 pfu (+: as virulent as parent virus; R: reduced by comparison with parent virus).

TABLE 5

List of double variants selected by sequential use of two monoclonal antibodies  
data of F. Gonzalez-Scarano, 1983

First Monoclonal Antibody	Second Monoclonal Antibody	Number of Variants
807-31	813-13	1
807-31	807-09	1
807-31	807-12	1
807-33	807-35	1
820-260	807-35	1
807-22	807-35	1
807-12	807-35	1

The single variants have been used for studies of virulence. Of the 11 variants tested (TABLE 4), two have shown attenuation in mice. One of these, V22, has been studied in some detail, as described below. Single variants have also been used for the mapping of epitopes, as described in the following section.

(i) Tryptic peptides of LAC and V22 viruses

Sequencing of the genome of monoclonal variants is now being done by primer extension and dideoxy chain termination with vRNA as the template. The M RNA is about 4.5 kb in length and encodes G1, G2, and a nonstructural (NSm) protein. Therefore, it would be useful to focus our sequencing to a shorter portion of the molecule. To this end we have been looking at tryptic peptides of a variant virus (V22) with reverse phase HPLC. Although the work is just beginning, the preliminary results are encouraging. V22 is chromatographed in parallel with LAC using double labels (14C for LAC and 3H for V22). The patterns are nearly identical, with one peptide present in LAC and absent in V22 and two peptides present in V22 but not in LAC (Fig. 1). If repeat experiments confirm this change, then the peptides can be isolated and sequenced by Edman degradation. This approach, which can be extended to other variants, would focus the sequencing to areas where antigenic changes should occur.

(j) Classification of epitopes on the G1 glycoprotein with monoclonal antibodies and variant viruses

The glycoproteins of enveloped viruses have several important biological functions including attachment to cellular receptors, fusion neutralization, and (in some instances) neuraminidase activity. It has been long established that the California serogroup of bunyaviruses agglutinate erythrocytes and are readily neutralized. Recently, we have shown (Gonzalez-Scarano et al, 1984) that these viruses can also mediate fusion from without or within. As an approach to further study of these glycoprotein functions, we isolated a panel of hybridomas and selected anti-G1 monoclonal antibodies (Gonzalez-Scarano et al, 1982). A subset of these antibodies mediated neutralization and hemagglutination-inhibition; since there was almost complete concordance between the two activities, it appeared that the same domain on the G1 protein was involved in attachment to BHK cells and erythrocytes. In addition, these antibodies appeared to define a second domain on the G1 protein which was not involved in either neutralization or HI activity.

In view of the importance of the biological activities of the G1 protein, it was of interest to determine whether the neutralizing monoclonal antibodies defined one or several antigenic sites. We elected to approach this question by testing a panel of variant viruses against the monoclonal antibodies used to select them (Najjar et al, 1985). A total of 35 variants, representing 11 different antibodies, were plaque-purified. These were then tested in two systems: (1) cross-neutralization of the variants against the panel of selecting monoclonal antibodies; and (2) an ELISA to compare the ability of antigens made from the parent La Crosse and the 11 variants to bind both neutralizing and non-neutralizing anti-G1 monoclonal antibodies.

Neutralization assays. Cross-neutralization tests were done with each of the 11 monoclonal antibodies against 1-6 variants per epitope. Representative results are summarized in Fig. 2. The results were scored as neutralization, partial neutralization, or no neutralization, as defined in the caption. With the exception of antibodies 807-12 and 807-33, all of the monoclonals showed distinct patterns of cross-neutralization. The neutralization antibodies fall into five distinct groupings (Table 4), with three of the groupings represented by a single antibody. The largest group, headed by antibody 807-

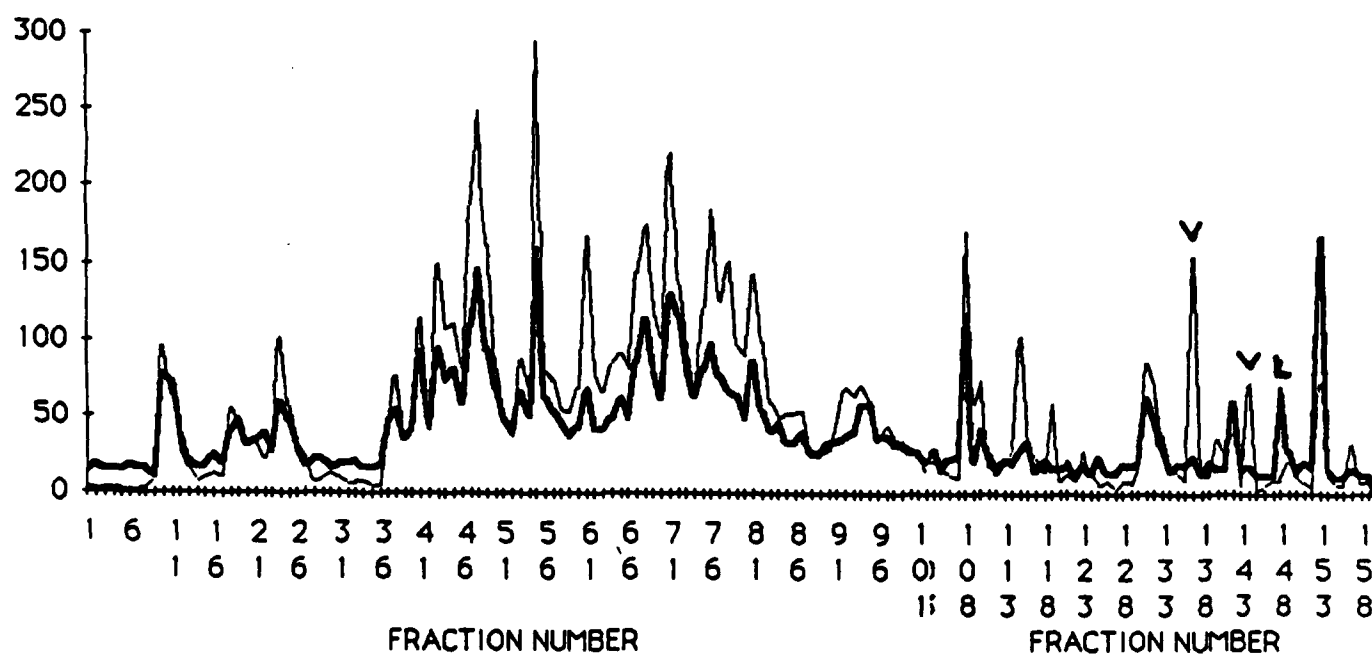


Fig. 1.

Reverse phase HPLC of tryptic peptides of LAC virus (heavy line,  $^{14}\text{C}$  label) and V2 virus (light line,  $^3\text{H}$  label) chromatographed together. There appear to be two unique peptides in V22 (V) and one in LAC (L) tryptic digests (F. Gonzalez-Scarano, unpublished, 1985).

31, is mainly composed of antibodies that are strain specific, and it represents the major neutralizing region on G1. Antibodies 807-12 and 807-33 appear identical by this analysis, but in fact showed differences when a panel of California viruses was used in neutralization assays with them (Gonzalez-Scarano et al, 1983).

ELISA. The 11 variant viruses (one variant per epitope) were tested against the 11 neutralizing monoclonal antibodies plus 8 non-neutralizing monoclonal antibodies against the G1 protein. Binding to parent LAC virus was defined as 100% and binding to variants was scored relative to this standard, after equalizing the amount of each variant used. Binding to variants ranged from 0% to 100% (or occasionally more than 100%), providing dramatic differences and the clear cut patterns in Fig. 3.

Almost all of the monoclonals showed significantly reduced binding by ELISA to the variant viruses selected with them; for most of the antibodies there was, in fact, no binding above background (Fig. 3). Antibodies 807-9 and 807-22 bound the variants selected with them when tested in ELISA, in spite of their inability to neutralize these variants. For antibody 807-22 this binding was only 69% of the binding parent LAC, but antibody 807-9 bound the variant selected with it as well as it bound parent LAC.

Many of the monoclonal antibodies also demonstrated reduced or no binding to variants selected with other antibodies, producing a pattern of interrelationships. The results obtained with ELISA are in excellent agreement with the findings of the neutralization tests (Fig. 2), although there are some differences with the map obtained in the cross-neutralization assays. There are five groups of antibodies that include at least one neutralizing monoclonal. The largest cluster, again headed by antibody 807-31, now consists of 9 monoclonals, since three non-neutralizing antibodies map to this area. The group headed by antibody 807-22 now also includes three non-neutralizing antibodies that show diminished binding to the variant (22) obtained with it. These four antibodies also demonstrate reduced binding to acid-treated virus (Gonzalez-Scarano, 1985). We had previously proposed that they comprise a cluster of epitopes which is affected by the conformational change that G1 undergoes at the pH of fusion.

Variant 25, which had appeared isolated in cross-neutralization assays, now appears closely related to variant 22, since it shows reduced binding of the four antibodies defining epitope 2. In addition, epitope 25 now is related to two other groups of antibodies (those headed by 807-31 and 807-12).

Comparison of neutralization and ELISA results. A comparison of the binding and cross-neutralization data is presented in TABLE 6. Over 85% (23/27) of the antibody-virus combinations that showed no neutralization also showed decreased binding in ELISA (0-74%). Similarly, 95% (88/92) of the antibody-virus combinations that neutralized showed binding on ELISA that was close to control values (75-100%).

Turning to apparent inconsistencies, there were four instances where neutralizing antibodies failed to neutralize variants to which they bound strongly (75%-100%), i.e., antibody 807-09 (variant 9), antibody 807-18 (variants 31 and 35), and antibody 807-15 (variant 13). A similar phenomenon is illustrated by the 8 non-neutralizing antibodies, most of which apparently bind to neutralizing epitopes. Similar observations (Roehrig et al, 1985) have been reported for other viruses, and there may be several explanations, such as low antibody-antigen affinity or multiple steps in the antibody-virus interaction leading to neutralization.

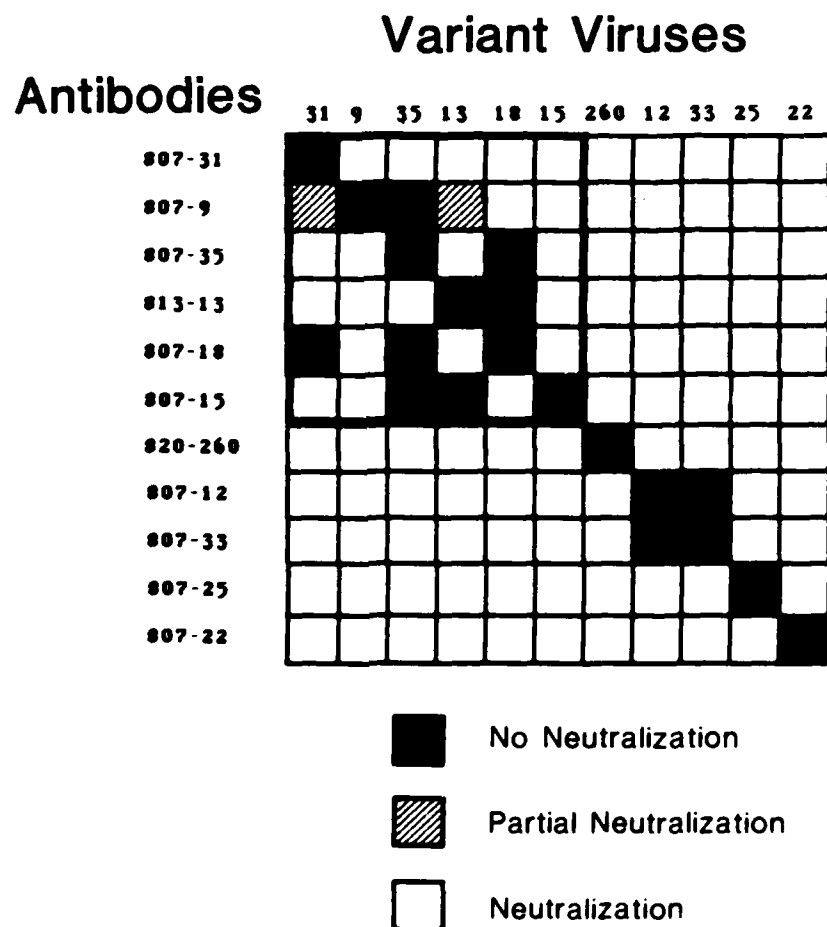


Fig. 2. Neutralization of antigenic variants by the monoclonal antibodies used to select them. The 11 antibodies shown neutralized parent La Crosse virus at titers ranging from 1:320 to 1:100,000. No neutralization was defined as the inability of an antibody to neutralize higher than 1:20. Neutralization was defined as a titer against a variant virus which was no less than 2-fold below the titer of the same antibody against parent La Crosse virus. Partial neutralization was seen only for antibody 807-09, as described in Najjar et al, 1985.



# Variant Viruses

## Antibodies

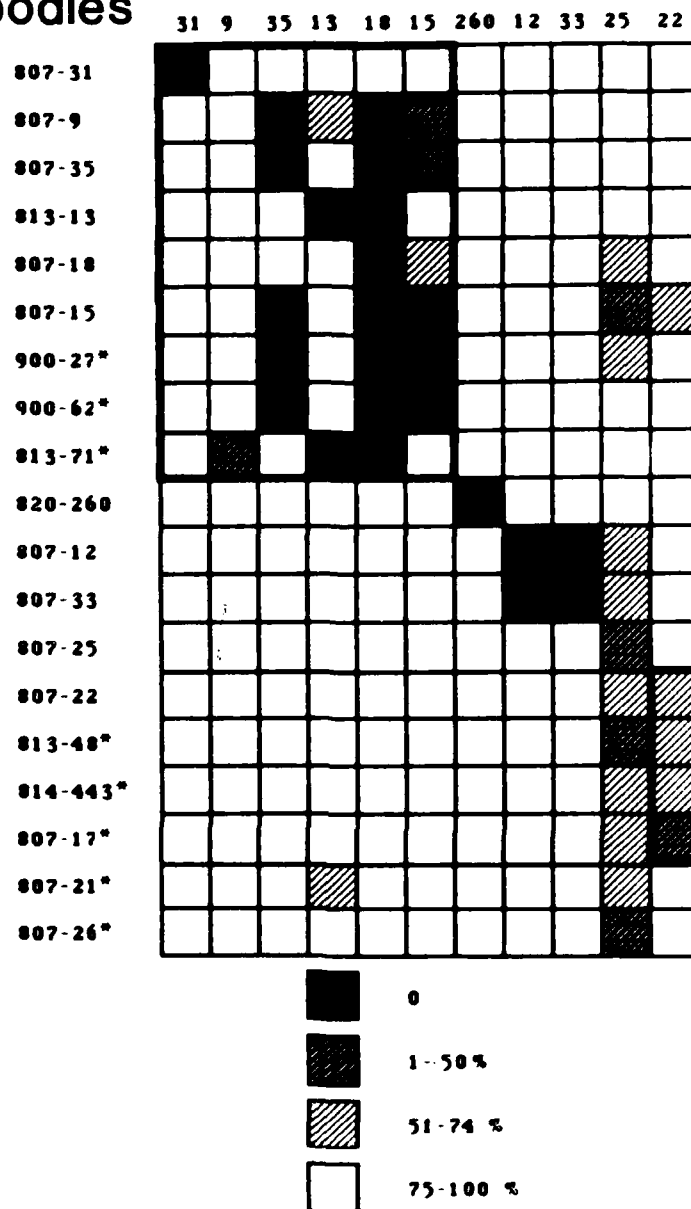


Fig. 3. Binding of anti-G1 glycoprotein monoclonal antibodies to antigenic variants in ELISA. Binding to variant viruses is recorded relative to binding of the same antibody to parent La Crosse virus. Values greater than 100% were grouped in the 75-100% category. Binding in the 1-50% category was almost always in the 20-50% range, with two exceptions. Asterisk (\*) indicates non-neutralizing antibodies.

TABLE 6

A comparison of neutralization and binding (ELISA) tests on 11 neutralizing monoclonal antibodies and the 11 variant viruses selected by them\*

Neutralization	ELISA			Totals
	0%	1-74%	75-100%	
None	14	9	4	27
Partial	0	1	1	2
Complete	2	2	88	92
Totals	16	12	93	121

\* Based on data in Figs. 2 and 3.

There are two instances where antibodies that neutralized a variant did not bind it on ELISA. That apparent inconsistency occurred only with variant 18 (and antibodies 807-9 and 807-15). It seems likely that the procedure used to coat virus onto ELISA plates, including alkaline pH, altered the conformation of epitope 18.

Summary. This analysis of the antigenic structure of the G1 glycoprotein of LAC virus leads to two major conclusions. (i) The epitopes identified by monoclonal antibodies can be separated into distinct groups or clusters. The neutralization assays define 5 such clusters, and the ELISA data suggest that there may be a sixth cluster defined by non-neutralizing antibodies 807-21 and 807-26. (ii) The data suggest that these 6 epitopes clusters may be a part of a single immunodominant antigenic site, since the ELISA results indicate interrelationships between all of the groups, with the exception of the single monoclonal antibody 820-260. Of particular importance, the data do not suggest that there is a separate non-neutralizing antigenic site as previously postulated (Gonzalez-Scarano et al, 1982; Kingsford and Hill, 1983).

(k) Protection of mice with monoclonal antibodies

One goal of our studies is to utilize monoclonal antibodies to analyze the epitopes involved in protective immunity against La Crosse and related bunyaviruses. We now have accumulated considerable information about our panel of antibodies against the G1 glycoprotein (TABLES 1 and 2): (i) neutralization titer or lack of neutralizing activity; (ii) grouping into epitopes of both neutralizing and non-neutralizing antibodies; (iii) hemagglutination-inhibition activity of antibodies, which correlates to a considerable extent with neutralizing activity; (iv) ability to block fusion, which also correlates to a considerable extent with neutralizing activity. It appeared timely to determine protective efficacy in mice.

We explored several alternative protocols for in vivo virus challenge to be used to screen for antibody mediated protection. It was considered desirable to (i) challenge by a peripheral route rather than by ic injection, in order to mimic a realistic challenge and to insure ability to detect protection; (ii) to use a modest virus dose to enhance ability to detect protection; (iii) to produce 100% mortality so that the screen could be carried out with a small number of mice; (iv) to administer antibody before challenge and by a different route to avoid an "in vivo neutralization test" and to mimic pre-exposure immunization. Trials of different challenges are set forth in TABLE 7. We concluded that the use of sc infection of suckling mice would best meet the foregoing criteria, with antibody given ip 1 day earlier.

We elected to screen a large number of antibodies first, rather than test a few antibodies in detail. The results of this screen are presented in TABLES 8 and 9. Several tentative conclusions can be drawn: (i) the test protocol works well and yields 100% mortality (0% protection) in controls, while protective antibodies give 0% mortality (100% protection). (ii) when tested in a single concentration of 1:8, clear cut results emerge which suggest that antibodies with neutralizing activity protect while those which are neutralization test negative fail to protect in vivo. (iii) there is no evidence that HI or FI activity is independently relevant to protection since antibodies which are N HI+ (814-443) fail to protect and antibodies which are N+ FI- (813-71) do protect. (iv) non-neutralizing antibodies which react with neutralizing epitopes 4 or 5 fail to protect (ie, 807-21 or 807-26). (v) individual monoclonal antibodies against single epitopes seem quite competent to mediate in vivo protection. Further studies which will be undertaken are described in a later section.

TABLE 7

Titration of La Crosse (LAC) virus by different routes in suckling and weanling  
CD-1 mice  
(V. Rentko, unpublished, 1984)

Age of Mice	Route of Infection*	Virus Dose in pfu ( $\log_{10}$ )	Per cent Mortality	$\log_{10}$ pfu per LD <sub>100</sub>
Weanling	ip	6.6	100%	5.6
		5.6	100%	
		4.6	25%	
		3.6	0%	
		2.6	0%	
	sc	6.6	75%	> 6.6
		5.6	50%	
		4.6	0%	
		3.6	0%	
	ic	6.4	100%	3.4
		5.4	100%	
		4.4	100%	
		3.4	100%	
		2.4	75%	
		1.4	0%	
		0.4	0%	
Suckling	sc	4.4	100%	1.4
		3.4	100%	
		2.4	100%	
		1.4	100%	
		0.4	0%	

\* 0.05 ml by either route.

TABLE 8

Protection of suckling mice given an ip dose of 0.05 ml of 1:8 concentration of monoclonal antibody and challenged 1 day later with 800 pfu (100 LD50) of La Crosse virus by the sc route (0.5 ml)

Protein Precipitated	Epitope Group	Antibody Number	Serotesting			Percent Protection
			NT*	HI	FI*	
GI	1	807-09	2.5	+	+/-	100%
	1	807-13*	1.0	+	-	0%
	1	807-15	2.8	+	+	100%
	1	807-18	3.4	+	++	100%
	1	807-31	4.0	+	+++	100%
	1	807-35	4.3	+	+++	83%
	2	820-260	4.0	ND	++	100%
	3	807-12	4.3	+	++	100%
	3	807-33	3.1	+	++	100%
	4	807-25	4.3	+	+++	100%
	5	807-22	2.5	+	++	17%
	4/5	813-48	3.7	+	+/-	67%
	1	813-71	2.5	ND	-	100%
	ND	813-77	2.5+	ND	-	100%
	1	900-27	2.8	ND	+/-	100%
	ND	807-01	1.0	ND	ND	0%
	ND	807-07	1.0	ND	ND	0%
	4/5	807-17	1.0	ND	-	0%
	4	807-21	1.0	-	-	0%
	4	807-26	1.0	-	-	0%
	ND	813-72	1.0	ND	ND	0%
	4/5	814-443	1.0	+	-	0%
	ND	900-11	1.0	ND	ND	0%
N		807-32	1.0	-	ND	0%
		814-02	1.0	-	ND	0%
		814-87	1.0	-	ND	0%
		820-260	1.0	-	ND	0%

\* For epitope grouping see TABLE 4. NT: neutralization tests run on same ascites tap tested for protection. HI: hemagglutination inhibition tests on a different ascites tap. FI: fusion inhibition test, where a single dilution of 1:50 was tested and the fusion index recorded as less than 0.40 (+++), 0.40-0.70 (++), 0.70-0.90 (+), 0.90 (+/-) or greater than 0.9 (-). Protection: based on mortality 2 weeks after challenge. 807-13: ascites fluid used for protection had no neutralizing activity although earlier taps were NT positive.

TABLE 9

Titration of protective monoclonal antibodies in suckling CD-1 mice, given 0.05 ml of antibody dilution ip and challenged by 1 day later with 800 pfu (100 LD50) of La Crosse virus, given as 0.05 ml by the sc route\*

Antibody Number	Antibody Concentration	Percent Protection
807-12	1:2	100%
	1:8	100%
	1:32	75%
807-33	1:8	100%
	1:32	100%
	1:100	100%
	1:500	75%
813-48	1:2	100%
	1:8	75%
	1:32	75%
813-77	1:8	100%
	1:32	75%
	1:100	0%
	1:500	0%

\* See footnote to TABLE 7.

(I) Next questions

The data reported above represent solid progress in the characterization of La Crosse and Tahyna hybridomas. However, there are major gaps which require further work. These include:

(i) Continuation of studies on grouping and mapping the G1 monoclonal antibodies. With the completion of epitope analysis of variant viruses, the focus will shift to physical mapping of the epitopes of the G1 protein. Two approaches are in progress. (a) cloning and sequencing the segment of the M RNA which encodes G1. This is a preliminary to sequencing variant viruses to localize those epitopes which represent sequential antigenic determinants. (b) Use of tryptic peptides of the G1 protein to localize differences in variant viruses. Alternatively, monoclonal antibodies could be tested for their reactivity with tryptic peptides. It will then be necessary to map antibody-reactive peptides onto the amino acid sequence of the G1 protein.

(ii) Construction of G2 hybridomas.

(iii) The use of monoclonal antibodies to determine the role of G1 and G2 proteins in fusion. Results to date indicate that G1 plays a critical role in fusion; the importance of G2 remains to be determined.

(iv) Determination of the potential protective role of neutralizing and non-neutralizing anti-G1 antibodies. Protection can now be correlated with neutralization, HI, and anti-fusion activities. Preliminary results indicate that all neutralizing monoclonal antibodies are potentially protective. It would now be possible to look in detail at selected monoclonal antibodies (perhaps column purified) to ask (a) whether protection is quantitatively related to plaque reduction titer; (b) whether protection is related to HI or FI activity; (c) whether protection relates to isotype; and (d) whether non-neutralizing antibodies can block protection conferred by neutralizing antibodies which bind to the same epitope.

(v) Ultimately, if some protective monoclonal antibodies can be mapped to specific tryptic peptides, it would be potentially possible to sequence such peptides, synthesize them, and test them as immunogens.

### 3. Virulence of La Crosse (LAC) and Tahyna (TAH) isolates

NOTE: As indicated above, we have designated the hybridoma studies as the specific object of this contract. However, the hybridomas will be used in experiments which are supported by our NIH grant NS 20904. Therefore, a brief account of the status of these experiments is set forth below.

The salient goal of our virulence and genetic studies is to correlate virus genes and their products (proteins) with biological properties of the virus, particularly virulence in rodents. The strategy which we are employing is to select and clone virus strains which demonstrate maximal differences in their virulence. The role of specific genes/gene products will then be analyzed in two ways: (a) construction of reassortant viruses using the selected clones as parents; (b) selection of hybridoma variant viruses, to look for the possible role of particular regions of the G1 molecule.

A further element in our plan is the selection of two viruses, La Crosse (LAC) and Tahyna (TAH), to use as parents in the construction of reassortants. This plan was based on the demonstration by Gentsch, Bishop and colleagues that LAC-TAH reassortants could readily be made. Also essential was the fact that antigenic differences between LAC and TAH permitted the use of monoclonal antibodies to distinguish glycoprotein (G1) and nucleocapsid (N) proteins of the two viruses. Furthermore, these two proteins of LAC and TAH viruses could also be distinguished by differences in their rate of migration in SDS-PAGE.

For our initial major genetic study we selected a LAC strain (LAC/original) which was virulent (neuroinvasive) in suckling mice and a TAH strain (TAH/181-57) which was avirulent (non-neuroinvasive). However, the only essential feature of the genetic strategy is the use of a LAC and a TAH strain which exhibit distinct biological properties.

#### (a) Standard LAC and TAH strains

As a first step in these studies, we wished to select two California serogroup viruses which differed dramatically in their virulence for mice after subcutaneous injection, which simulates mosquito transmission.

To confirm that the standard strains of LAC (original) and TAH (Bardos 92) would exhibit characteristics similar to those published by Shope and others, we did age-specific titrations with the results shown in TABLE 10. These titrations were consistent with the published literature and confirmed that by the ip route, TAH virus is nonlethal in mice age 2 weeks or older. By contrast, LAC virus will kill older mice, although it requires 10,000 - 100,000 pfu to kill 50 - 100% of 4-week-old mice.

These results reinforced our original presumption that it would be preferable to have a LAC clone which showed greater ip virulence in weanling or adult mice or a TAH clone which showed less ip virulence in sucklings. This goal was achieved with the selection of an attenuated strain, TAH/181-57, described below. The LAC/original strain was used as a virulent prototype since it represented an isolate from the brain of a fatal human case.



TABLE 10  
Titrations of LAC and TAH viruses in mice

Assay	Log <sub>10</sub> Titer per ml*		
	LAC/ori	TAH/B92	TAH/181-57**
pfu	7.2	ND	7.1
ic LD50			
1-3 days	7.1	6.3	7.3
3-4 weeks	6.5	6.2	ND
8-10 weeks	5.8	4.5	ND
ip LD50			
1-3 days	6.2	6.1	2.8***
1 week	ND	3.0	ND
2 weeks	ND	1.0	ND
3-4 weeks	2.8	1.0	ND
8-10 weeks	2.0	1.0	ND

\* LAC: LAC/original in BALB/c mice.  
TAH: TAH/B92 and TAH/181-57 in outbred CD mice.  
Undiluted: supernate of a 10% brain homogenate.  
ND: not done.

\*\* Data of R. Janssen, 1982.

\*\*\* Subcutaneous injection.

(b) Selection and comparative pathogenesis of an avirulent (non-neuroinvasive) strain: TAH/181-57

Review of the literature indicated that the avirulent 181-57 strain of Tahyna (TAH/181-57) was much less virulent than the standard B92 strain of Tahyna, originally reported by Malkova. TAH/181-57 was then obtained from Malkova and adopted as the prototype avirulent virus. TABLE II shows that, although one pfu was equal to one intracerebral LD50 for both viruses, the sc LD50 was 20 pfu for LAC/original virus and 20,000 pfu for TAH/181-57 virus.

Experiments in suckling mice (Janssen et al, 1984) employed two different subcutaneous doses. At the lower dose (700 pfu) LAC/original killed all mice while (in most experiments) TAH/181-57 killed none. A large dose (475,000 pfu) was also used; since both viruses killed mice at this dose, an additional comparison of pathogenesis could be made.

After sc infection with 700 pfu of LAC/original, mice remained healthy until the third day; they then became less active, parietic, pale, and died by 4 days. At this dose of TAH/181-57 virus, mice usually remained asymptomatic. After infection with 475,000 pfu of LAC/original virus, mice underwent the same sequence of signs, but died one day earlier (medium survival, 2.5 days). At this large subcutaneous dose TAH/181-57 also killed, but signs evolved more slowly and the median survival time was 4.7 days.

To follow the course of infection, mice were sacrificed at regular intervals following infection and selected tissues were titrated for virus content. LAC/original virus was isolated from all the extraneural tissues tested (muscle, heart, liver, and spleen) but the highest titers were in muscle and titers in other tissues fell below the level in plasma. Titers in brain paralleled but exceeded those in any other tissue by at least 100-fold, reaching levels of  $10^{7.5}$  pfu per mg. Following sc injection of 700 pfu, TAH/181-57 showed no evidence of replication in muscle or plasma, and was not detected in brain.

At the large sc inoculum (475,000 pfu) both viruses killed mice, but there were still considerable distinctions between virulent and avirulent strains. LAC/original showed an accelerated course of replication compared with the low dose of the same virus. Again, muscle titers exceeded those in other tissues, most of which fell below the viremia level. Brain titers surpassed those in extraneural tissues reaching peak levels of  $10^{7.5}$  pfu per mg tissue. Following sc inoculation of 475,000 pfu, TAH/181-57 replicated much more slowly than virulent LAC/original. Furthermore, maximal titers in muscle, the key extraneural tissue, were 100 fold-lower than those for LAC/original.

Immunofluorescent staining was used to follow the accumulation of viral antigen in peripheral and CNS tissues during the course of infection in suckling and adult mice. In suckling mice inoculated sc, the salient question was the sites of extraneural virus replication. A great many observations indicated that striated muscle was the major site of peripheral replication. Suckling mice given a sc dose of 475,000 pfu of TAH/181-57, a lethal inoculum, showed much less evidence of extraneural replication than was produced by either dose of LAC/original virus.

The CNS of suckling mice showed a very similar picture regardless of route of infection or of virus strain. The major features of CNS infection were: (1) The overwhelming nature of infection, such that many areas of brain could be described as a "sea of fluorescence". (2) Neurons were the main target, and white matter tracts, such as corpus callosum and cord showed strikingly less fluorescence. (3) All neuronal nuclei,

TABLE II

Titers of LAC/original and TAH/181-57 viruses in suckling CD-1 mice, expressed as pfu per LD50\*

Route	LAC/original	TAH/B92	TAH/181-57
intracerebral		1	1
intraperitoneal		6	3
subcutaneous		17	ND

\* Based on single representative experiments, with 8-20 mice per dilution. ND: not done.

together with grey matter of cord, appeared susceptible. (4) Even though the peripheral infectivity of virulent and avirulent viruses differed, they produced an indistinguishable picture within the CNS.

Comment. The distinction between neuroinvasive LAC/original and non-invasive TAH/181-57 was clear and striking: virulence was associated with replication in striated muscle and production of a high titer viremia. These differences are accentuated by the use of an avirulent (non-neuroinvasive) strain of Tahyna virus which permitted studies in suckling mice.

The characteristics of TAH/181-57 were undoubtedly associated with its history of over 50 brain-to-brain passages. Malkova indicated that this strain resembled other Tahyna strains shortly after isolation and only acquired its low invasiveness after multiple brain passages.

(c) Other virus strains with different biological characteristics: LAC/PP-31 strain

The strategy we have employed to study avirulent TAH/181-57 virus can also be applied to other determinants of virulence. To this end, we are collecting or selecting additional strains with major differences in biological properties. One of these is described briefly below.

PP-31: mosquito-attenuated strain of LAC virus. See: Miller, BR. A variant of La Crosse virus attenuated for Aedes triseriatus mosquitoes. Am J Trop Med Hyg 32:1422-1428, 1983. Dr. Barry Miller has kindly given us PP-31, a clone of LAC/original virus. This clone differs from LAC/original in its inability to initiate productive infections in Aedes triseriatus mosquitoes. PP-31 apparently infects midgut cells but fails to spread to salivary gland or ovary; therefore this strain is not transmitted through the vector to a new vertebrate host or transovarially to progeny mosquitoes.

If this strain retains the peripheral virulence for mice characteristic of LAC/original, then it may define a determinant of mosquito infectivity different from peripheral mouse virulence (neuroinvasiveness). Genetic crosses of PP-31 with a standard virulent TAH (strain B92) could be used to identify the viral RNA segment responsible for mosquito infectivity. If the M RNA segment is responsible, then PP-31 may identify a genetic determinant differing from the determinant responsible for peripheral mouse virulence, but one which is encoded in the same RNA segment.

(d) Construction of reassortants from non-mutagenized parents: phenotyping and genotyping

For genetic studies of virulence (Janssen et al, 1985) it was deemed advisable to avoid the use of reassortants constructed from ts mutants, since these reassortants have "silent" (non-ts) mutations in all their genes. Furthermore, experience with both bunyaviruses (laboratory of D.H.L. Bishop) and reoviruses (laboratory of B. Fields) has shown that reassortants made from ts mutants may show unpredictable aberrations in their biological properties due to "silent" mutations. Two methods for phenotyping were used, SDS-PAGE of the viral proteins and ELISA with monoclonal antibodies. These permitted the rapid phenotyping of large numbers of plaques from dually infected cultures.

BHK-21 monolayers on 24 well-plates (Costar) were co-infected with LAC/original and TAH/181-57 viruses at several multiplicities (TABLE 12). After 30 minutes, the inoculum was removed and the plates incubated at 33C for 18-24 hours. Each well was harvested and later titrated in a standard plaque assay. Individual plaques were grown in BHK-21 monolayers to make mini-pools.

Phenotyping by polyacrylamide gel electrophoresis. A single well in a 24 well COSTAR plate containing a BHK monolayer, was infected at an MOI of 1.0, incubated 16 hours, pulse labelled for 1.5 hours with 35S methionine, and lysed with Laemmli's sample buffer. The cell lysate was run on reducing SDS-PAGE and fluorographed. The G1 and G2 of LAC/original migrate more rapidly, while the nucleocapsid (N) protein of LAC/original migrates more slowly than the corresponding proteins of TAH/181-57. PAGE was used to phenotype the 291 clones shown in TABLE 12.

Phenotyping by ELISA with monoclonal antibodies. An alternative approach to rapid phenotyping is the use of monoclonal antibodies against the G1 and N proteins of LAC and TAH viruses. By selecting antibodies which are relatively specific for each virus it is possible to do a rapid "pattern" test. The method for preparing ELISA antigens and conducting the test has been published (Gonzalez-Scarano et al, 1982). When ELISA is applied to 7 selected reassortants (TABLE 13) the patterns confirm the tentative conclusions from PAGE.

Genotyping the RNA segments. Phenotyping has the serious limitation that it does not permit identification of the L protein. Therefore, we turned to genotyping, to type the L RNA and to confirm the phenotyping of M and S RNAs. We have modified a method of RNA-RNA hybridization, originally introduced by Alan Hay for influenza virus, to genotype our reassortants (Janssen et al, 1985). Two RNA preparations are made: (i) P32 labelled virion RNA (vRNA\*) from purified virions, and (ii) unlabelled virus complementary RNA (vcRNA) from lysates of infected cell cultures harvested 6 hours after infection. RNA is phenol extracted and ethanol precipitated. Hybridization employs vRNA\* from parental LAC or TAH viruses (90,000 cpm) plus vc RNA from each clone to be genotyped (yield of 3/5 of 1 10cm Petri dish). As shown in Fig. 5, this method gives unambiguous results.

A panel of reassortants, shown in TABLE 14, was then assembled using the methods described above. A total of 13 reassortants was composed of 1-3 representatives of each of the 6 possible reassortant genotypes.

Virulence of reassortants. To obtain a quantitative assessment of the 13 reassortants, each virus was titrated by the subcutaneous (sc) route in suckling CD-1 mice; the pfu titer was divided by the sc LD50 titer to yield an index (pfu per LD50) of virulence.

Incomplete results are summarized in TABLE 14. Those reassortants with a La Crosse M RNA segment show a virulence similar to that of parent La Crosse virus. There is one exception, clone B1-10a, which may be a spontaneous mutant since it has a small plaque phenotype, not seen in either parent virus.

Reassortants with a Tahyna M RNA segment show considerably greater virulence than parent Tahyna virus, and can be classified as intermediate between the two parental strains. Data are not sufficiently complete to know whether the L RNA as well as the S RNA segments (from La Crosse virus) can modulate the effect of the Tahyna M RNA segment.

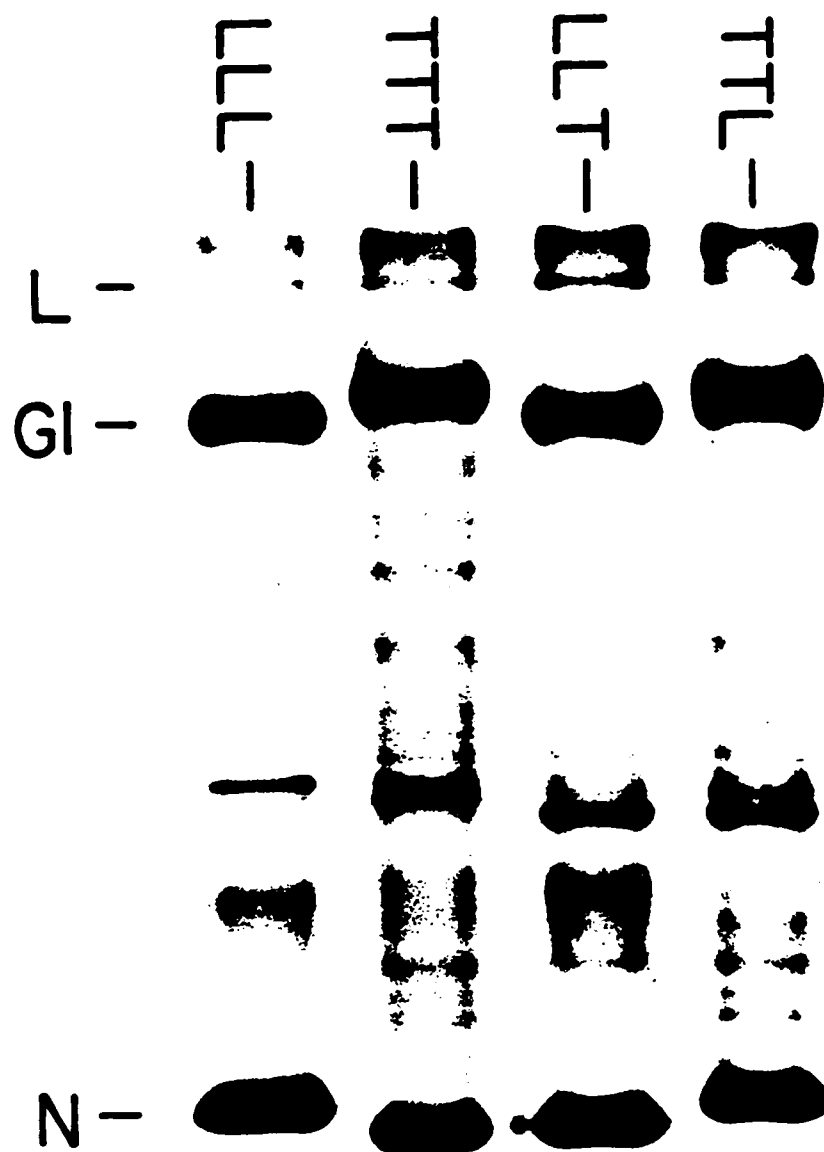


Fig. 4. SDS-PAGE in 12.5% acrylamide of proteins of S35 methionine-labelled banded virions. The 4 lanes show the two parental viruses (LAC, LLL; TAH, TTT) and two reassortants of known genotype. The G2 protein is poorly labelled and runs ahead of a non-virion band which is probably actin.

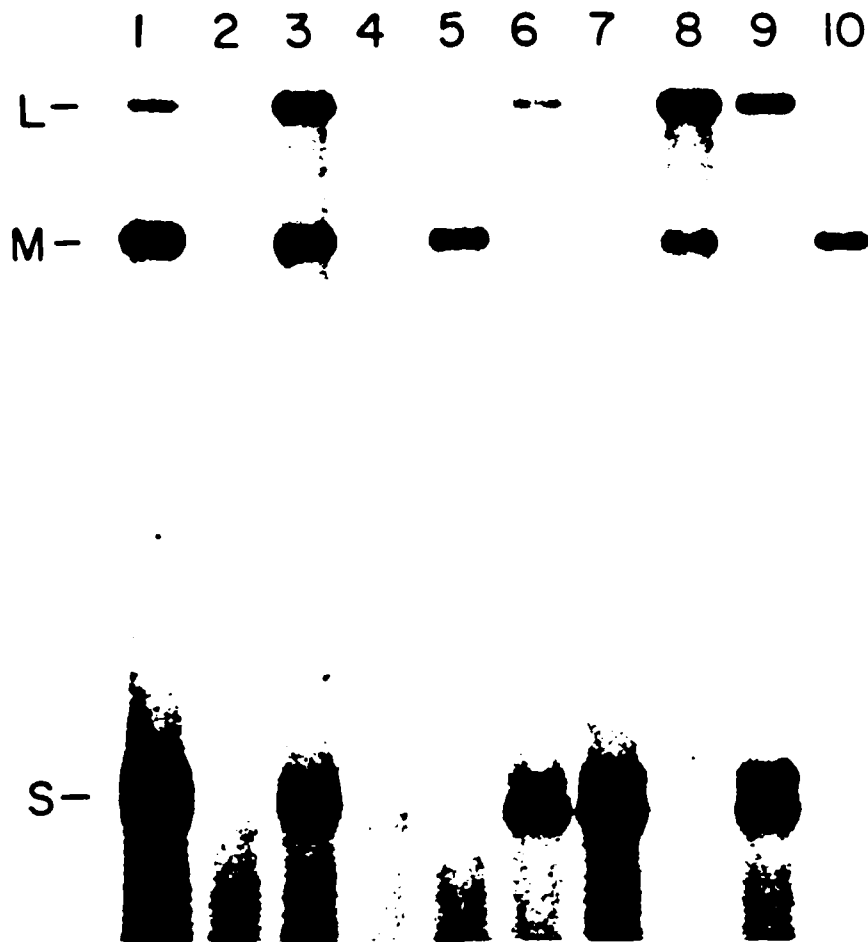


Fig. 5. RNA-RNA hybridization for the genotyping of reassortants between LAC and TAH viruses. Each virus to be tested was prepared as a vcRNA, from unlabelled cell lysate. Each vcRNA was hybridized with vRNA\* from LAC and from TAH viruses, prepared from P32 labelled banded virions. The L, M, and S RNA segments are marked.

Lane	vcRNA	vRNA*
1	LLL	LLL
2	TTT	LLL
3	TTT	TTT
4	LLL	TTT
5	TLT	LLL
6	TLT	TTT
7	TTL	LLL
8	TTL	TTT
9	LTL	LLL
10	LTL	TTT

TABLE 12

Summary of reassortants generated by co-infections between LAC/original and  
TAH/181-57 viruses

Experiment No.	MOI LAC/TAH	Number of Reassortants by Phenotype					
		Total	XLL	XTT	XXX	XTL	XLT
A1	0.5/0.5	26	18	00	6	0	2
B1	0.5/2.0	28	16	07	1	0	4
E1	25/25	06	05	00	1	0	0
F1	5/5	20	03	10	2	2	3
F2	5/5	28	01	12	8	2	5
F3	5/5*	14	00	11	0	0	3
F4	5/5*	21	01	18	0	1	1
G1	5/10	16	04	11	0	0	1
G2	5/10	24	03	18	1	0	2
G3	5/10	20	04	12	2	0	1
G4	5/10*	24	00	20	0	0	4
H1	5/25	04	00	04	0	0	0
H2	5/20	27	00	18	4	0	5
H3	5/25*	12	00	10	2	0	0
H4	5/20*	21	00	21	0	0	0
Totals		291	55	172	17	5	31

\* Monoclonal antibody 807-31 (0.1 ml of 1:1000 dilution) against LAC/original virus (type-specific) was added to these infections. Phenotyping was done by PAGE. MOI: multiplicity of infection.



TABLE 13

Mortality and survival following subcutaneous infection of suckling mice with 700 pfu of parental LAC/original and TAH/181-57 viruses or of reassortant clones

Virus Clone	Phenotype***	Mortality	Virulence*
Parents	LLL	100%	+
	TTT	0-17%	-
AI-3a	XLT	100%	+
BI-11a	XLT	100%	+
BI-26a	XLT	100%	+
BI-29a	XLT	100%	+
FI-2a	XTL	0%	-
FI-18a	XTL	0%	-
BI-10a	XLT	70-100%	+/-**

\* Virulence as based on subcutaneous injection of suckling mice with 700 pfu. This dose of LAC/original is usually 100% lethal, while the same dose of TAH is usually 0% lethal.

\*\* Prolonged survival time and small plaques.

\*\*\* Phenotyped by both PAGE and ELISA.

TABLE 14

Mortality and survival following subcutaneous infection of suckling mice with parental LAC/original and TAH/181-57 viruses or of reassortant clones  
data of R. Janssen, 1985

Virus Clone	Phenotype	Genotype	PFU/LD50 sc injection	Virulence
LAC/ori	XLL	LLL	14	+
TAH/181	XTT	TTT	20,000	-
A1-3a	XLT	LLT	8	+
B1-29a	XLT	LLT	14	+
B1-11a	XLT	TLT	2	+
B1-26a	XLT	TLT	2	+
B1-10a*	XLT	TLT	711	+/-
P1-26b+	XLL	TLL	?	?
P1-13c+	XLL	TLL	?	?
F1-2a	XTL	TTL	1473	+/-
F2-2a	XTL	TTL	?	?
F1-18a	XTL	LTL	245	+/-
F2-18a	XTL	LTL	7535	+/-
F4-5a	XTL	LTL	?	?
B1-1a	XTT	LTT	?	?

\* Possibly a spontaneous mutant, since it produced small plaques as well as prolonged survival of mice.

+ Obtained from a cross of TTL and TLT.

Comments. Shope and Bishop (1981) reported that, within the California serogroup, neuroinvasiveness co-segregates with the M RNA segment. The major new finding which emerges from the present study is the polygenic determination of virulence. It appears that, with reassortants possessing the avirulent M RNA, the presence of one or both of the other segments from La Crosse virus confers an intermediate virulence which is distinctly greater than that of the avirulent Tahyna 181-57 parent. Apparently, this modulating effect can be conferred by the S RNA and perhaps also by the L RNA segment. The assay system is not sufficiently precise to show whether there is an additive effect of these two genes.

In sum, these studies indicate that the M RNA segment plays a dominant role in the determination of virulence. The middle RNA segment of bunyaviruses encodes the two envelope glycoproteins G1 and G2 (Bishop and Shope, 1979). These proteins mediate the first two steps in infection, that is, attachment to cellular receptors and fusion between the viral envelope and a host membrane (Gonzalez-Scarano et al, 1982, 1984). Alterations in either of these functions could influence replication in key target cells, such as myocytes, and thereby regulate virulence. Independent evidence that the G1 protein does indeed play a role in virulence comes from recent work (Gonzalez-Scarano et al, 1985) showing that selected variant viruses, representing mutants in the G1 protein, have reduced virulence.

#### (e) Avirulence of selected variant viruses

The study of virus variants selected with monoclonal antibodies can provide a powerful approach to the delineation of the molecular basis of virus virulence, since such variants may have reduced virulence. In the rabies system the genome of the variants with decreased pathogenicity has been sequenced. As with other monoclonal variants, the alteration is a single non-redundant base substitution, leading to a single amino acid change, but the biological function that is inhibited has not yet been studied. Several reovirus variants selected with a single monoclonal antibody show a dramatic decrease in virulence when injected intracerebrally in suckling mice, and it has been postulated that this decrease in pathogenicity is due to altered receptor binding.

Variants of La Crosse (LAC) virus were selected with neutralizing monoclonal antibodies directed against the major glycoprotein, G1. The variants were present at an average frequency of  $10^{-5.4}$ , and those for the site by antibody 807-22, at a frequency of  $10^{-6.3}$  (TABLE 4). After plaque purification, 1000 plaque-forming units (pfu) were inoculated subcutaneously into suckling mice (Gonzalez-Scarano et al, 1985). Unlike the parent virus and most of the variants selected by other monoclonal antibodies, V22 was unable to kill 100% of the mice. The number of PFU required to kill 50% of mice (LD50) inoculated with V22 was determined for suckling, weanling, and adult outbred mice (CD-1, Charles River) after intraperitoneal and intracerebral inoculation (TABLE 15). When compared with LAC virus in parallel experiments, there is a reduction in mortality following peripheral inoculation of V22 of 100- to 1000-fold. After intracranial inoculation of V22, the LD50 is diminished about 25-fold, indicating that although the salient biological defect is reduced ability to penetrate the CNS, there is also reduced neuronotropism.

Detailed studies of pathogenesis (Gonzalez-Scarano et al, 1985) showed the reduced neuroinvasiveness of V22 to be associated with reduced viremia and reduced replication in skeletal muscle (Fig. 6). Replication in the brain, by contrast, was only minimally retarded.

TABLE 15

Virulence of La Crosse (LAC) virus and of variant 22 (V22) virus in CD-1 mice  
expressed as log<sub>10</sub> pfu per LD50\*

Route of Injection	Virus	Age of Mice		
		Suckling	Weanling	Adult
Intracerebral	LAC	-0.3	0.3	0.52
	V22	0.9	1.77	1.97
Intraperitoneal	LAC	0.49	3.71	3.90
	V22	2.35	6	7

- \* A tissue culture stock of each virus, with a titer of  $2 \times 10^8$  PFU/ml was used for all experiments. Swiss albino mice (CD-1), were inoculated with the appropriate volume (i.c. 0.02 ml, i.p. 0.05 ml) of virus diluted in 0.75% bovine albumin/PBS. Five weanling or adult mice were inoculated per ten-fold dilution. Suckling mice experiments used one litter/dilution.

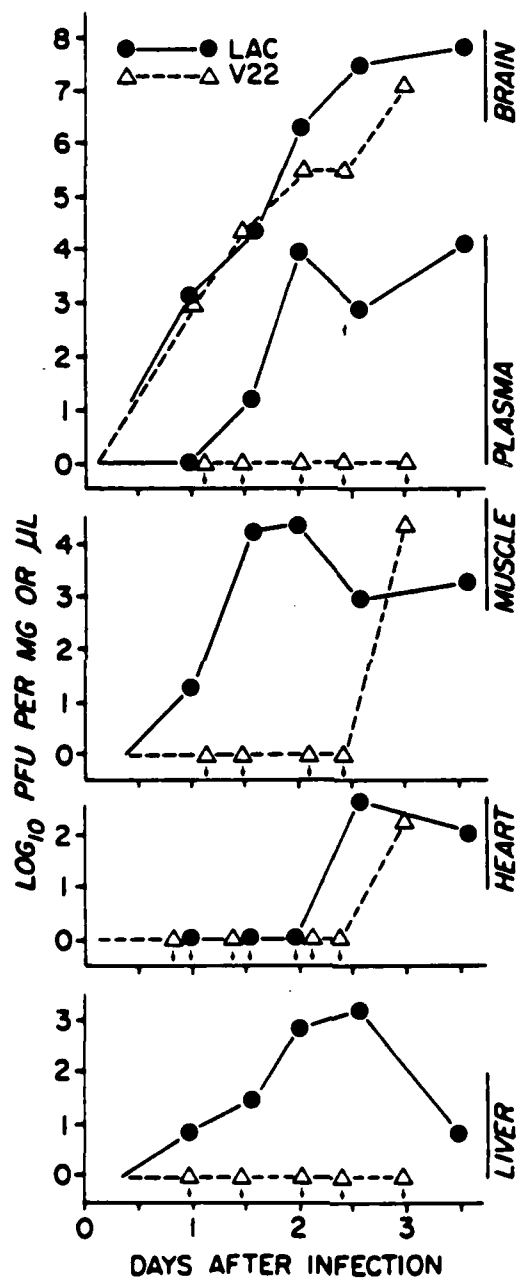


Fig. 6. Replication of La Crosse/original (LAC) and variant 22 (V22) viruses in suckling CD-1 mice after intraperitoneal injection of 2200 pfu. Each point represents a pool of tissues from 3 mice.

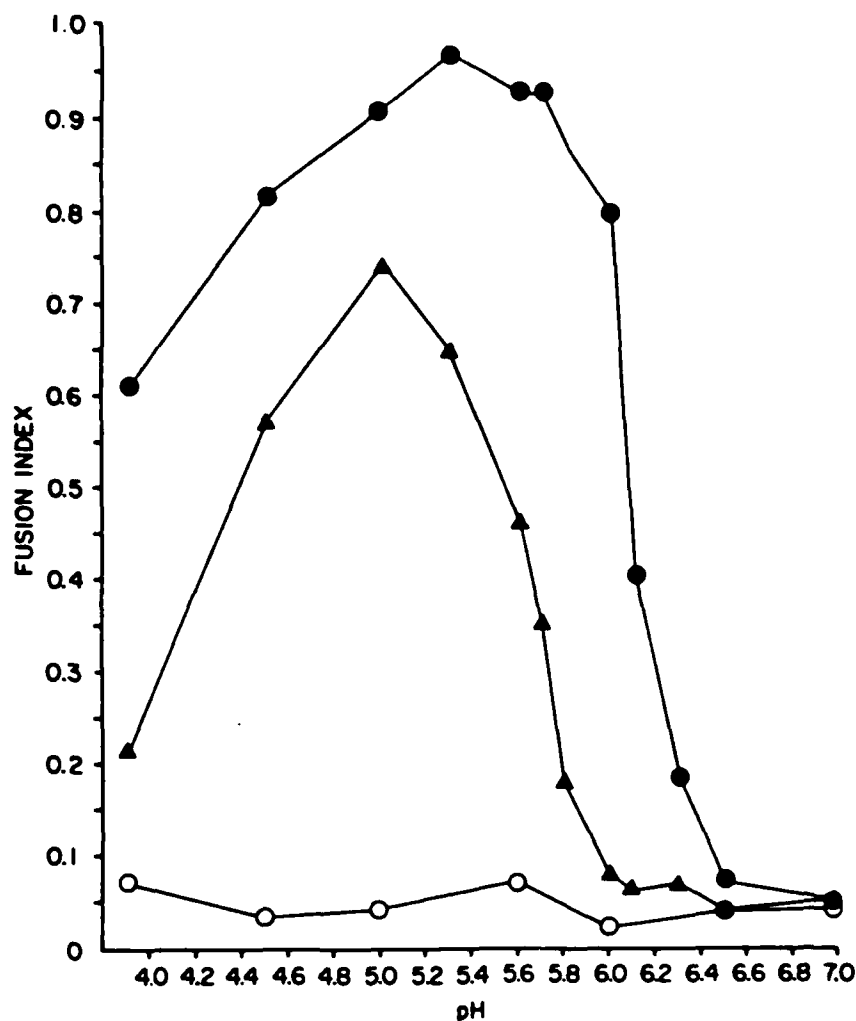


Fig. 7. Fusion from within by LAC and V22 viruses. A sub-confluent monolayer of BHK-21 cells on 96-well (COSTAR) plates ( $2.2 \times 10^4$  cells/well) was inoculated with LAC or V22 at moi of 1.0, kept at room temperature for one hour, washed with PBS, then incubated with MEM + 2% FCS at 35C. Sixteen hours after infection the cells were washed with PBS, exposed to MEM at the appropriate pH, and incubated for 30 minutes. The cells were then fixed, stained and the fusion index (1-(no. of cells/mo. of nuclei)) determined after counting the nuclei and cells (circa 1700 nuclei per data point). The 95% confidence limit for all points was  $\pm 0.05$ . ●: LAC; ▲: V22; ○: uninfected control.

V22 grew in baby hamster kidney (BHK-21) cells at a rate equivalent to that of parent virus, but peak titers were usually 0.5 log<sub>10</sub> lower than for LAC, regardless of the initial multiplicity of infection (MOI). When growth under agar was compared, the V22 plaques were noticeably smaller.

Since these data suggested that the deficiency responsible for decreased virulence was probably not poor interaction with cellular receptors, we looked at another major glycoprotein function. We have recently shown that LAC virus is capable of fusing BHK-21 cells when absorbed at 4°C and exposed to pH 6.0 (fusion from without, FFWO). Fusion from within (FFWI), that is, fusion of cells that have been actively infected, also occurs with LAC, and the pH characteristics are similar to FFWO. V22 was able to mediate FFWI, but the pH of optimal fusion was lower (5.1 rather than 5.4), and the maximal efficiency of fusion (fusion index, FI, 0.7), even at optimal pH, was lower than the fusing efficiency of LAC (FI, 0.95), as shown in Fig. 7.

A comparison of FFWO showed that the maximum level of fusion with the variant is lower than with LAC (FI, 0.65 vs 0.84). The concentration of virus required for 50% maximal fusion was 0.5 µg/20 µl for LAC and 4.0 µg/20 µl for V22, ie, maximal fusion with V22 required 8 times the protein required for maximum fusion with LAC virus.

The demonstration that a monoclonal variant deficient in the fusion function has altered neuroinvasiveness implies that fusion plays an important role in the extraneural phase of infection with LAC. Our work with this virus, and with naturally occurring non-neuroinvasive strains, indicates that high levels of replication in muscle are essential for neuroinvasiveness. The altered fusion function could retard the initial steps in the infection of individual cells, since a low pH step has been postulated in the entry pathway of enveloped viruses, or it could reduce the efficiency of cell-to-cell spread of virus. Regardless of the mechanism, the results emphasize the importance of the viral envelope proteins and all their functions in determining virus virulence.

(f) Next questions

Our studies of virulence are now well under way, and the program has entered a period of consolidation.

(i) LAC/original virus variants (35) selected with the 11 monoclonal antibodies must be characterized as to virulence and as to fusion, to determine the consistency with which each epitope affects virulence and fusion.

(ii) Multiple epitope variants of LAC/original should be selected and characterized as to virulence, to determine the effect of multiple mutations.

(iii) It is important to select additional biological variants, for further genetic studies. We are now attempting to select a variant with reduced neurovirulence on intracerebral injection, to compare genetic control of neurovirulence and of neuroinvasiveness. Other biological variants, such as PP-31, require evaluation in mice.

(iv) It will then be possible to conduct genetic studies with a few (1 or 2) selected biological variants, to map properties such as neurovirulence.

(v) A more rapid method for genotyping, such as blot hybridization with synthetic oligonucleotides would be very useful.

(vi) Variants of TAH/181-57 or a few other biological variants, should be selected and characterized to determine whether they exhibit increased virulence.

(vii) A myotube cell culture system should be explored to see if it reflects the in vivo differences between TAH/181-57 and LAC/original viruses.

The more exploratory aspect of our virulence studies will focus on mapping the responsible epitopes onto the viral genome, specifically onto the M RNA segment. To this end, a stepwise approach is planned, including:

(viii) Cloning or obtaining a clone of the M RNA segment.

(viii) Determination of the nucleotide sequence of the M RNA segment and of the amino acid sequence of the NH<sub>4</sub> terminus of G1, making it possible to infer the amino acid sequence of the G1 protein.

(ix) Mapping the G1 mutation sites of a few selected variants, emphasizing those with biologically interesting changes. This can be done either by direct sequencing of virion M RNA segment or by identifying alterations in the tryptic peptides of the G1 protein.



# Appendix I

## La Crosse Bunyavirus Can Mediate pH-Dependent Fusion from Without

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Lipid binding properties which are dependent on exposure to acid pH are an important mechanism for the cellular entry pathway for some enveloped viruses and for other macromolecules. Cell-to-cell fusion can be used to demonstrate this function. La Crosse virus, a member of the family Bunyaviridae, fused BHK-21 cells from without (FFWO) upon exposure of the absorbed virus to pH 6.3 or below. A high multiplicity of infection and temperature of 37° were necessary for optimum fusion. The pH requirement was similar to that reported for Semliki forest virus, an alphavirus, but higher than that required for the demonstration of cell fusion by members of the Orthomyxoviridae. Virus inactivated by ultraviolet light also mediated FFWO. This is the first report of pH-mediated fusion for this family of viruses and it suggests, by analogy with other viruses, that their entry pathway includes exposure to acid.

Over the past few years, many enveloped viruses have been shown to have lipid binding and membrane fusing properties which become manifest only upon exposure of the virions to acidic pH (1, 2). These properties may be necessary in order for virions to extrude their nucleocapsids into the cellular cytoplasm following uptake by endocytosis into acidic prelysosomal vacuoles which have been called receptosomes or endosomes by different groups (3-5). Other macromolecules, like toxins, may be discharged into the cytosol by a similar mechanism (6, 7).

The interaction of portions of viruses with membranes prior to uncoating involves spike glycoproteins and, presumably, hydrophobic portions of these molecules. Among the RNA viruses, low pH-mediated fusion has been demonstrated for vesicular stomatitis virus (VSV), a rhabdovirus, Semliki forest virus (SFV), an alphavirus, and for members of the Orthomyxoviridae family (8, 9). Among them, the fusion mechanism for the influenza viruses has been studied best, and it is known to require post-translational cleavage of precursor hemagglutinin (HA0) to HA1 and HA2 and appears to be mediated by a conformational change of the HA (10).

It had been noted previously that the amino terminus of HA2, consisting of 10 highly conserved uncharged amino acids, was analogous to the F1 component of Sendai virus fusion glycoprotein (11, 12).

Although fusion of virion envelopes with cellular membranes can be visualized with the electron microscope (13), routine demonstration by this method is difficult. Therefore, a variety of indirect measures of fusion have been introduced to characterize the parameters of this glycoprotein function. Among these, low pH-mediated hemolysis of red blood cells (2, 9) and cell-to-cell fusion of tissue culture cells (8, 9) are the simplest. Cell-to-cell fusion may employ virus absorbed on cells (fusion from without (FFWO)) or viral glycoproteins expressed on the surface of infected cells (fusion from within (FFWI)). We report here the fusion of BHK-21 cells (FFWO) mediated by La Crosse virus (LAC). This is the first report of FFWO by any member of the family Bunyaviridae.

Cell fusion was demonstrated by adaptation of the method of White *et al.* (8). BHK-21 clone 13 cells were grown in 60-well Terasaki (Costar) plates to a density of  $9.6 \times 10^4$  cells/cm<sup>2</sup> using MEM with 10% FCS. After removal of the growth medium,

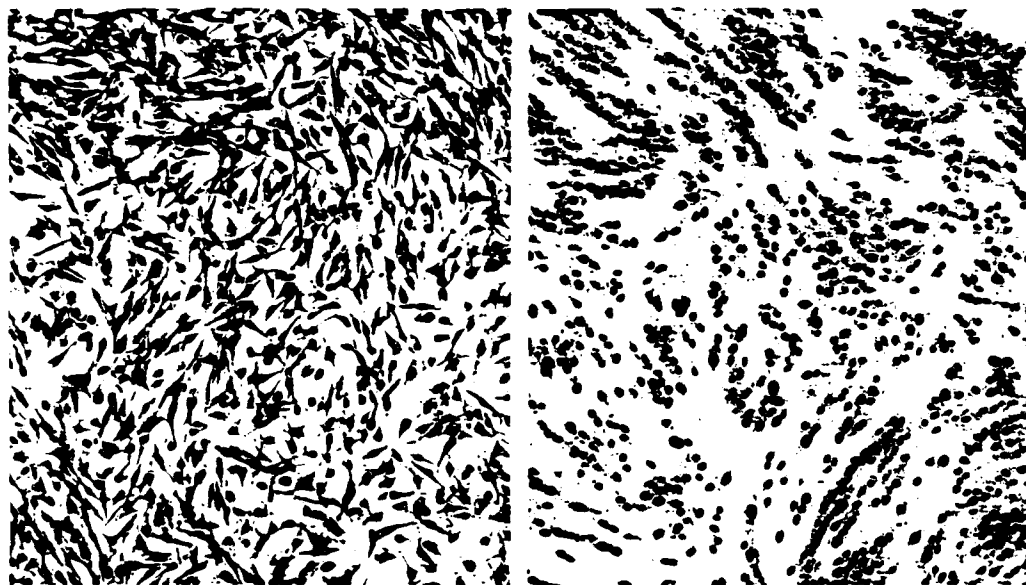


FIG. 1. BHK-21 clone 13 cells were grown to a density of  $9.6 \times 10^4$  cells/cm<sup>2</sup> in treated toxoplasmosis titer plates with Eagle's MEM with 10% fetal calf serum. LAC virus was grown in BHK-21 cells and precipitated from clarified tissue culture with 7% polyethylene glycol 8000 in 362 mM NaCl, banded in a 20-70% sucrose gradient in 1 M NaCl, 10 mM Tris, pH 7.4, pelleted, and resuspended in 0.15 M NaCl, 10 mM Tris, pH 7.4 (LSB). It was added to the cells at a concentration of 0.2  $\mu$ g/ $\mu$ l and allowed to absorb for 1 hr at 4°. The inoculum was then removed and the cells were briefly (30-60 sec) exposed to prewarmed MEM with 0.2% BSA and 10 mM MES buffer at pH 7.0 (left) or pH 5.8 (right). The pH buffer was removed and the cells were incubated at 37° under neutral pH for 30 min, after which the monolayer was washed with PBS, fixed, and stained with Giemsa stain. (Final magnification,  $\times 85$ .)

band-purified (legend, Fig. 1) LAC was added (6  $\mu$ g in 20  $\mu$ l) and maintained at 4° for 1 hr, following which the inoculum was removed. The cells were briefly (30-60 sec) exposed to prewarmed MEM with 0.2% BSA containing 10 mM Hepes and 10 mM morpholinopropanesulfonic acid (MOPS) or morpholinoethanesulfonic acid (MES) buffer adjusted to the appropriate pH with NaOH. The buffer was removed and the cells were incubated at 37° under neutral pH for 30-60 min, after which the monolayer was fixed and stained with Giemsa (Difquick, Am. Sci. Prod.) stain. Cell fusion occurred to greater or lesser extent at pH 5-6.3, depending on the time of incubation. Above pH 6.3 cell fusion was not observed, even after incubation for 80 min. Figure 1 shows FFWO at pH 5.8.

The fusion index (1 - (No. of cells/No. of nuclei)) for all experiments was determined (8) by directly counting 100 nuclei

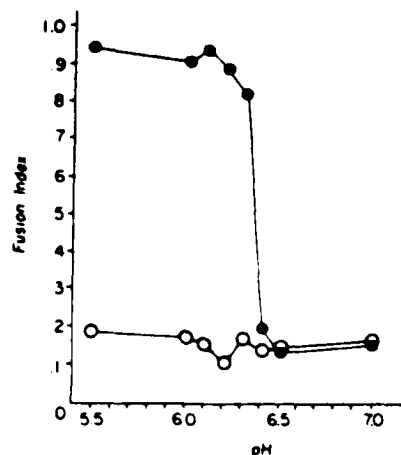


FIG. 2. Cells and virus were prepared as described in the legend to Fig. 1, but the cells were grown in 60-well Terasaki plates (Costar) and exposed, following absorption of virus (●) or of LSB (○), to buffers of different pH. The fusion index (1 - (No. of Cells/No. of nuclei)) was calculated by counting 800 nuclei per data point.

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in representative fields of duplicate wells in two or three identical experiments. At 37° and 30 min, maximum fusion was obtained at pH 5.0-6.0 (Figs. 2, 3). The pH range at which LAC-mediated BHK-21 fusion occurs is similar to the range reported for SFV (8).

The amount of virus used in each experiment was critical in influencing the extent of fusion (Fig. 4). The differences in technique and cell type make comparisons difficult, but the microgram concentration of virus necessary for maximum fusion with LAC was larger than that reported for SFV (8). The particle/PFU ratio has not been determined for the bunyavirus group, and the figure of 2600 PFU/cell for maximum fusion is consistent with the data for SFV, which has been reported to require 3000 viruses/cell, in a smaller volume, for optimal fusion of BHK-21 cells (8). The temperature at which the cells were incubated following exposure to low pH was important also (Fig. 3). At low temperatures, cell fusion does not proceed at all, and it is slow at 31.5°. This effect cannot be overcome by increasing the amount of virus bound to cells or by prolonging the incubation time. Fusion also

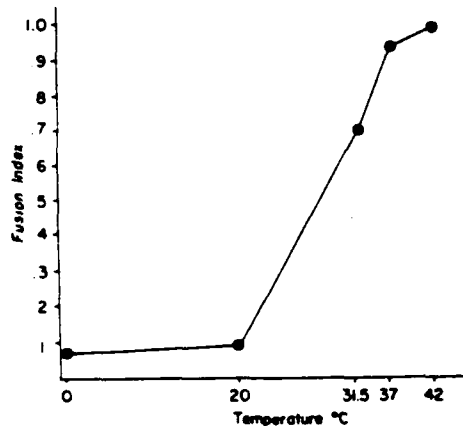


FIG. 3. Temperature dependence of fusion. Virus (7.8  $\mu$ g), prepared as described in the legend to Fig. 1, was absorbed on BHK-21 cells at 4° for 1 hr. The cells were then briefly exposed to MEM-MES buffer, pH 5.8, at the temperatures indicated, and, after replacement of the buffer with neutral MEM, incubated at that temperature for 30 min. The fusion index was calculated as described in the legend to Fig. 2.

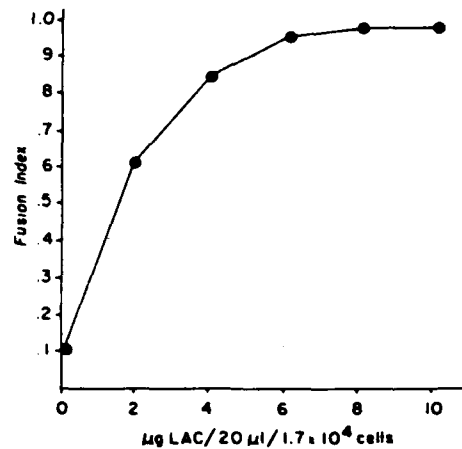


FIG. 4. Effect of increasing concentration of virus on FFWO. Concentrations of purified virus (see Fig. 1) were measured after disruption in 0.1% Triton-X with the Bio-Rad method, using BSA as a standard. The virus was absorbed in a volume of 20  $\mu$ l/Terasaki well for 1 hr at 4°, and the cells were exposed to pH 5.8 buffer, neutralized, and incubated at 37° for 30 min. The fusion index was calculated as described in the legend to Fig. 2.

occurs with virus that has been inactivated by exposure to ultraviolet light to reduce the PFU titer by 100,000-fold (data not shown).

LAC virus has two envelope glycoproteins, G1 (120 kDa) and G2 (34 kDa) (14, 15), which are present in approximately equimolar amounts. Either molecule, or both, could be involved in FFWO. Our preliminary data suggest that G1 undergoes a conformational change at acidic pH, altering both tryptic cleavage and antigenic sites, but in the absence of sequence information, it is not possible to predict whether it, or G2, contains the fusion peptide.

By analogy with other systems (13, 16, 17), the demonstration that LAC has a pH-dependent fusion function extends the putative entry pathway involving acidic vesicles to another family of enveloped viruses.

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# Mechanisms of Bunyavirus Virulence

## Comparative Pathogenesis of a Virulent Strain of La Crosse and an Avirulent Strain of Tahyna Virus

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To analyze mechanisms of virulence in the California serogroup bunyaviruses, the virulent La Crosse/original (LAC/original) strain was compared with the avirulent Tahyna/181-57 strain. In suckling mice, both viruses were lethal upon intracerebral injection but differed markedly in their neuroinvasiveness following subcutaneous injection; 20 and 20,000 plaque-forming units, respectively, were equivalent to 1 subcutaneous LD<sub>50</sub>. The sequential course of infection was followed after subcutaneous injection of 700 plaque-forming units; LAC/original replicated in striated muscle, caused a high titer plasma viremia, invaded the central nervous system, and killed all mice; the same dose of avirulent Tahyna/181-57 failed to replicate in extraneural tissues, did not invade the central nervous system, and caused no apparent illness. Immunofluorescent examination of peripheral and central nervous system tissues showed the same distinctions between virulent and avirulent viruses and pinpointed striated muscle as the major extraneural target of virulent LAC/original virus. Paradoxically, after intracerebral injection of suckling or adult mice, Tahyna/181-57 virus killed more quickly than LAC/original. This difference was correlated with replication differences; Tahyna/181-57 multiplied marginally faster in the brain than did LAC/original virus.

**Additional key words:** California serogroup virus, California encephalitis, Acute viral encephalitis.

Bunyaviruses offer an attractive model to study the molecular mechanisms of viral pathogenesis for several reasons. First, the virion is composed of only four structural polypeptides; a large (L) protein presumed to act as an RNA polymerase, two glycoproteins (G1 and G2), and a nucleocapsid protein associated with the viral RNA (2). The negative-strand genomic RNA occurs in three segments (L, M, and S RNAs); the M and S RNAs encode, respectively, the G1 and G2 and the nucleocapsid proteins. Among the members of the California serogroup of bunyaviruses, genetic reassortants can be constructed, and this makes it potentially possible to examine the role of individual genomic segments as determinants of the biologic properties of the virus.

Second, La Crosse virus (a member of the California serogroup) when injected into mice provides an excellent model for California encephalitis in humans. The mouse develops acute encephalitis or asymptomatic infection, and the outcome can be altered by manipulating virus dose, route of infection, and age of the animals.

As a first step in these studies, we selected two California serogroup viruses that differed dramatically in their virulence for mice after subcutaneous (sc) injection, which simulates mosquito transmission. The La Crosse/original (LAC/original) strain was used as a virulent prototype since it represented an isolate from the brain

of a fatal human case (20). A Tahyna virus was sought as the avirulent prototype because it was known (5) that under experimental conditions genetic reassortment could occur between La Crosse and Tahyna viruses. In addition Tahyna virus differs sufficiently from La Crosse virus in both electrophoretic migration of proteins in acrylamide gels and in antigenicity; therefore, it is possible to distinguish between the G1, G2, and nucleocapsid proteins of the two viruses. Since the reference strain of Tahyna (Bardos 92 strain) is only slightly less virulent in suckling mice than the LAC/original strain, an attenuated Tahyna strain (181-57) was obtained (12, 13) that would facilitate analysis of the differences in pathogenesis.

The present study compares the pathogenesis of LAC/original and Tahyna/181-57 (TAH/181-57) in suckling mice under conditions in which the virulent virus kills mice, whereas the avirulent virus causes only a subclinical asymptomatic infection. Very clear differences in pathogenesis are seen, and they appear to be associated with differential ability to replicate in striated muscle.

### MATERIALS AND METHODS

#### VIRUSES

LAC/original, isolated from the brain of a girl who died of encephalitis in Wisconsin (20), was subsequently

passed nine times brain-to-brain in suckling mice. Following two plaque purifications on BHK-21 cells, a working stock was prepared from a tissue culture supernatant; the titer of this stock was  $10^{8.4}$  plaque-forming units (pfu) per ml.

TAH/181-57 was isolated from *Aedes vexans* mosquitoes in Czechoslovakia and passed 57 times brain-to-brain in suckling mice (12, 13). It was then plaque purified twice on BHK-21 cells, and a working stock was prepared from clarified tissue culture supernatant; the titer of this stock was  $10^{7.9}$  pfu/ml.

#### ANIMALS

Outbred albino Swiss mice (CD-1; Charles River Breeding Laboratories, Inc.) were used in all experiments, except for preliminary titrations in which BALB/c (Flow Laboratories, Inc.) were used.

#### TISSUE TITRATIONS

To follow the replication of virus, mice of various ages were infected with calibrated inocula, by intracerebral (ic) injection of 0.02 ml or sc injection of 0.05 ml. At various intervals, mice were sacrificed by cervical dislocation or decapitation, and selected tissues (brain, quadriceps muscle, heart, liver, spleen, blood) from three mice were pooled and prepared as 10% suspensions in minimal essential medium with 2% fetal calf serum. Ten-fold dilutions were made in minimal essential medium with 2% fetal calf serum and assayed by plaquing on BHK-21 cells using an overlay of 0.5% agarose in minimal essential medium with 2% fetal calf serum. Plaques were read at 72 hours, and titers were recorded as plaque-forming units per milligram of tissue or microliters of plasma, with a sensitivity of 1 pfu/mg or  $\mu$ l.

#### IMMUNOFLUORESCENT OBSERVATIONS

Blocks of mouse tissue were frozen at  $-80^{\circ}\text{C}$  and sectioned in a cryostat at  $6\text{ }\mu\text{m}$ , air dried, fixed in acetone at  $4^{\circ}\text{C}$ , air dried, and stored at  $-20^{\circ}\text{C}$  until examination.

The indirect immunofluorescent method was used. A hyperimmune rabbit anti-LAC or anti-TAH antiserum (neutralization titer 1:3200) was employed in the first step, and a fluorescein isothiocyanate-conjugated goat antirabbit IgG (light and heavy chain specific, N. L. Cappel Laboratories, Inc.) was employed in the second step. Both reagents were used at a dilution of about 1:16, and Evans blue (final concentration, 0.5% v/v) was added to the conjugate as a counterstain. Staining procedures followed published methods (6), and sections were examined by epiillumination in a Zeiss photomicroscope, with a 490-nm exciter filter and a 520-nm barrier filter. Brains were cut in parasagittal section, and infection was recorded with a semiquantitative grading system (4) for different areas of the brain (see footnote to Table 3). Controls for specificity were regularly included; the only nonspecific staining seen was confined to chondrocytes, cartilage, skin, and mucosa.

#### HISTOLOGIC PREPARATIONS

Mice were perfused, and tissue was stored in Bouin's solution, embedded in paraffin, and stained with Luxol fast blue and hematoxylin and eosin.

## RESULTS

### AGE-SPECIFIC TITRATIONS OF LA CROSSE AND TAHYNA VIRUSES

The relative virulence for mice of La Crosse and Tahyna viruses was defined by age-specific titrations by ic and intraperitoneal (ip) routes. The data are recorded in Figure 1 according to the number of plaque-forming units required to kill 50% of animals ( $\text{LD}_{50}$ ). The general pattern, as reported by Johnson and Johnson (9), is that suckling mice are highly susceptible to small virus doses injected by any route; with increasing age there is a modest reduction in ic susceptibility and a dramatic reduction in susceptibility to virus inoculated by ip or sc routes. Based on ip titrations, the prototype La Crosse strain (LAC/original) was only slightly more neuroinvasive than the prototype Tahyna strain (B92), and this difference was only seen in mice aged 2 to 4 weeks. Therefore, the avirulent TAH/181-57 was tested and shown to have low neuroinvasiveness as originally reported by Malkova (12, 13). TAH/181-57 was then adopted as the prototype avirulent virus.

All further experiments employed suckling mice (infected within 3 days of birth), since there were dramatic differences in the peripheral virulence of LAC/original and TAH/181-57 in these animals. Table 1 shows that, although 1 pfu was equal to 1 ic  $\text{LD}_{50}$  for both viruses, the sc  $\text{LD}_{50}$  was 20 pfu for LAC/original virus and 20,000 pfu for TAH/181-57 virus.

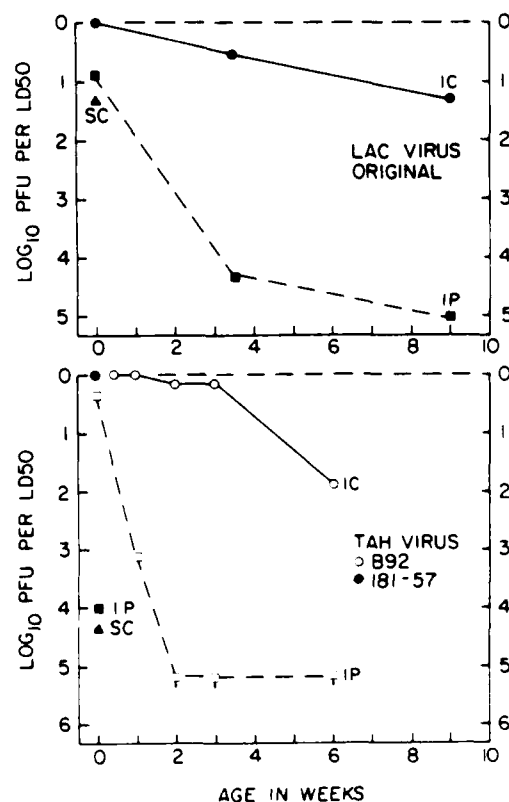


FIG. 1. Age-specific titrations of LAC/original and of Tahyna virus, strains Bardos 92 (B92) and strain TAH/181-57 in mice. All TAH titrations were performed in CD-1 mice; LAC titrations performed in CD-1 mice (sucklings) or BALB/c mice (all ages). ic, 0.02 ml of inoculum; ip, 0.05 ml of inoculum; sc, 0.05 ml of inoculum.

## SUBCUTANEOUS INFECTION OF SUCKLING MICE

Experiments in suckling mice employed two different sc doses (Table 2). At the lower dose (700 pfu) LAC/original killed all mice, whereas (in most experiments) TAH/181-57 killed none. A large dose (475,000 pfu) was also used; since both viruses killed mice at this dose, an additional comparison of pathogenesis could be made.

After sc infection with 700 pfu of LAC/original, mice remained healthy until the third day; they then became less active, parietic, and pale and died by 4 days. At this dose of TAH/181-57 virus, mice usually remained asymptomatic. After infection with 475,000 pfu of LAC/original virus, mice underwent the same sequence of signs but died 1 day earlier (median survival, 2.5 days); sc TAH/181-57 virus also killed at this large dose but signs evolved more slowly, and the median survival time was 4.7 days.

To follow the course of infection, mice were sacrificed at regular intervals following infection, and selected tissues were titrated for virus content. The levels are recorded as plaque-forming units per milligram with the sensitivity of the assay being 1 pfu/mg. The top panel of Figure 2 shows the results after infection with 700 pfu. LAC/original virus was isolated from all of the extraneural tissues tested (muscle, heart, liver, and spleen), but the highest titers were in muscle, and titers in other tissues decreased to less than the level in plasma. Titers in brain paralleled but exceeded those in any other tissue by at least 100-fold and reached levels of  $10^{7.5}$  pfu/mg. Following sc injection of 700 pfu, TAH/181-57 showed no evidence of replication in muscle or plasma and was not detected in the brain.

At the large sc inoculum (475,000 pfu), both viruses killed mice, but there were still considerable distinctions between virulent and avirulent strains (Fig. 2). LAC/

original showed an accelerated course of replication compared with the low dose of the same virus. Again, muscle titers exceeded those in other tissues, most of which decreased below the viremia level. Brain titers surpassed those in extraneural tissues; they reached peak levels of  $10^{7.5}$  pfu/mg of tissue. Following sc inoculation of 475,000 pfu, TAH/181-57 replicated much more slowly than virulent LAC/original. Furthermore, maximal titers in muscle, the key extraneural tissue, were 100-fold lower than those for LAC/original. With this indolent course, it was possible to see that the viremia peaked at 2.5 days, prior to replication in the brain. Viremia actually decreased to minimal levels at 6 and 7 days, prior to the death of mice. Likewise, virus did not appear in the brain until 3.5 days after infection and plateaued at about  $10^6$  pfu/mg, clearly lower than seen in overwhelming infections with either virus.

## IC INFECTION OF SUCKLING AND ADULT MICE

LAC/original and TAH/181-57 have high neurovirulence in suckling mice, as judged by titrations which indicate (Table 1) that ic injection of 1 pfu of either virus will kill 50% of suckling mice. To examine neurovirulence further, suckling mice were injected ic with an inoculum of 700 pfu, and survival curves and virus replication in brain were followed. LAC/original always killed mice more slowly than did TAH/181-57 (Table 2). This observation presented an apparent paradox; that is, LAC/original killed mice more rapidly than TAH/181-57 after sc injection but more slowly after ic injection. The explanation emerges from the data in Figures 2 and 3, which clearly show that, following ic injection, LAC/original replicates marginally more slowly than TAH/181-57, whereas following sc injection LAC/original replicates much more rapidly than TAH/181-57 (Fig. 2).

Since neither virus produces viremia in adults, these animals provide an uncomplicated comparison of neurovirulence. Figure 3 shows that, as in suckling mice, LAC/original replicates more slowly in the brain of adult mice than does TAH/181-57.

## IMMUNOFLUORESCENT OBSERVATION OF VIRAL ANTIGEN

Immunofluorescent staining was used to follow the accumulation of viral antigen in peripheral and central nervous system (CNS) tissues during the course of infection in suckling and adult mice. This was particularly important for the localization of target cells in extraneural tissues in which the viremia made interpretation of tissue titrations ambiguous. Selected observations are illustrated in Figures 4 through 6 and summarized in Table 3. Viral antigen was apparently confined to the cytoplasm in all tissues in which the nucleus could be identified microscopically.

**Extraneural Tissues.** In suckling mice inoculated sc, the salient question was the sites of extraneural virus replication. A great many observations (Table 3) indicated that striated muscle was the major site of peripheral replication, and a typical focus is shown in Figure 4. Striated muscle showed multiple foci of infection, which appeared when the tissue titers reached about  $10^3$  pfu/mg and became larger and more frequent as the titers

TABLE 1. TITERS OF LAC/ORIGINAL AND TAH/181-57 VIRUSES IN SUCKLING CD-1 MICE\*

Route	LAC/ original	TAH/ B92	TAH/ 181-57
ic	1	1	1
ip	6	3	10,000
sc	17	ND	20,000

\* Expressed as plaque-forming units per LD<sub>50</sub>. Data based on single representative experiments, with eight to 20 mice per dilution. ND, not done.

TABLE 2. SURVIVAL TIMES OF SUCKLING MICE INFECTED WITH LAC/ORIGINAL OR TAH/181-57 VIRUSES ACCORDING TO ROUTE OF INJECTION, VIRUS INOCULUM, AND VIRUS STRAIN\*

Route of injection	Virus inoculum pfu	Virus strain	Mortality	Median survival time
			%	da
sc	700	LAC	100	3.0
		TAH	0	—
	475,000	LAC	100	2.5
		TAH	100	4.7
ic	700	LAC	100	2.8
		TAH	100	1.8

\* Mortality based on five to 10 separate experiments for each set of variables. Survival based on single experiments with about 40 animals each; —, no deaths.

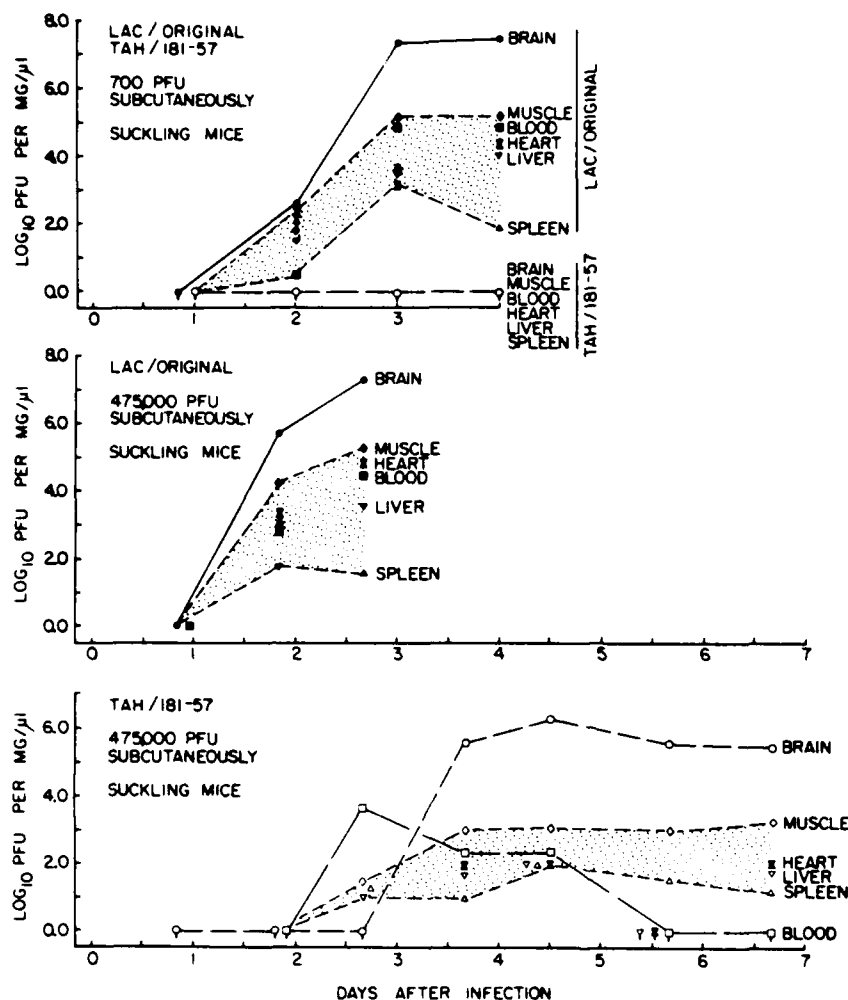


FIG. 2. Replication of LAC/original and TAH/181-57 viruses following sc injection in suckling CD-1 mice. *Top panel*, Dose of 700 pfu. At this dose LAC kills all mice, and TAH produces an inapparent immunizing infection. *Middle and bottom panels*, Dose of 475,000 pfu. At this dose both viruses kill mice. Stippled zone indicates the range of virus titers in extraneural tissues. Titers in plaque-forming units per milligram of tissue or per microliter of plasma. Each data point represents tissue pooled from three mice.

rose. Occasionally, cardiac muscle, parasympathetic plexus in gut, and (rarely) renal tubular cells showed fluorescence. We did not note any antigen in either vascular endothelium or in chondrocytes.

Suckling mice given a sc dose of 475,000 pfu of TAH/181-57, a lethal inoculum, showed much less evidence of extraneural replication than was produced by LAC/original virus (Table 3). Striated muscle was the major or exclusive site of visible antigen.

**CNS.** CNS of suckling mice showed a very similar picture regardless of route of infection or of virus strain. However, as shown in Table 3, the conditions of infection did influence the extent of involvement. Following a lethal sc injection of LAC/original virus, antigen first appeared in ependymal cells, striatum, and rhinencephalon at 48 hours; in moribund mice 3 to 4 days after infection, antigen was widespread. Mice given a large sc dose of TAH/181-57 showed a similar distribution of antigen in CNS, although it was less extensive, consistent with the lower virus titers (Fig. 2).

The major features of CNS infection in suckling mice were (a) The overwhelming nature of infection, such that many areas of brain could be described as a "sea of fluorescence" (Fig. 4 illustrates cerebral cortex). (b) Neurons were the main target, and white matter tracts, such

as corpus callosum and cord, showed strikingly less fluorescence (illustrated for adults in Fig. 6), although it was not possible to rule out infection of various glial elements. (c) All neuronal nuclei, together with gray matter of cord, appeared susceptible. Involvement of dorsal root ganglia, periaortic ganglia, and the submucosal plexus of gut suggested centrifugal neuronal dissemination. (d) Even though the peripheral infectivity of virulent and avirulent viruses differed, they produced an indistinguishable picture within the CNS.

Adult mice showed a distinctly different picture of CNS infection than did suckling mice. The involvement was much more discrete, so that individual infected cells could be clearly ascertained (Fig. 5). Again, infection involved most areas of the gray matter of the brain (Table 3), with marked sparing of white matter (Fig. 6).

#### PATHOLOGIC LESIONS IN BRAIN

Histologic examination was limited to the brains of moribund mice. In suckling mice, the presence of lesions was markedly influenced by survival time. Mice dying within 3 days postinfection were remarkable for the paucity of identifiable changes when the overwhelming nature of the infection is considered. More than half of the mice dying 4 to 6 days after infection had necrotic



fields of cells in cerebral cortex (Fig. 4) or less often in rhinencephalon, hippocampus, thalamus, or midbrain tectum. Within necrotic areas there were sparse infiltrates of mononuclear cells and minimal perivascular cuffs.

Adult mice also showed only limited brain lesions, confined to the pyramidal cell layer of the hippocampus, which showed zones of necrosis in most moribund mice (Fig. 6). Only a few inflammatory cells were seen in such lesions, and there was almost no evidence of perivascular

cuffing or other inflammatory hallmarks of viral encephalitis.

Adult mice also showed a second striking lesion. This was one or several sharply demarcated areas of focal spongy softening with very little inflammation (not illustrated). Such lesions were confined to rostral midbrain and hypothalamus, were seen in about two-thirds of moribund mice, and were not related to the site of inoculation.

## DISCUSSION

### RECONSTRUCTION OF SEQUENTIAL STEPS IN PATHOGENESIS

There is a body of work on the pathogenesis of La Crosse and related viruses in the mouse (1, 9, 21, 23) that permits a reconstruction of the sequential steps in infection (see Johnson (10) for a general discussion). The present study sheds light on several of these steps.

1. An active viremia appears to be essential for CNS invasion. At moderate sc inocula (700 pfu), the ability to initiate an active viremia appears to be the critical difference between neuroinvasive LAC/original and non-invasive TAH/181-57 viruses. When a massive dose (475,000 pfu) of TAH/181-57 is injected by the sc route, active viremia appears at 2.5 days and precedes CNS replication, which is delayed until 3.5 days. This indicates that the passive viremia that follows injection is not adequate to deliver TAH/181-57 virus to the CNS, in spite of the fact that only 1 pfu of the virus can initiate lethal infection after direct CNS injection.

2. In these studies the source of viremia, clearly appeared to be virus that had replicated in striated muscle. We did not detect viral antigen in vascular endothelium, in agreement with Wallnerova (23), in most other extra-neural tissues. How virus is transported from muscle to blood is unknown, but it may well be via lymphatic drainage into the circulation. Several other investigators working with California serogroup viruses (1, 23) or with alphaviruses (8, 11, 14) have described experimental infections in which striated muscles were the major source of viremia.

3. Previous workers (9, 17) have described circulating virus as a plasma viremia, and the present observations are consistent with this view. There was no evidence of viral antigen in spleen, lymphoid tissues, or bone marrow.

4. The mechanism by which virus is transported across the blood-brain barrier is unclear (10); as noted earlier we failed to observe endothelial infection (reported by Johnson and Johnson (9) but not seen by Wallnerova (23)) or early involvement of choroid plexus or other vascularized CNS membranes. Since 1 pfu is sufficient for the initiation of CNS infection, it may be impossible to observe the movement of virus across the barrier.

5. In addition to dissemination through the blood, La Crosse and Tahyna viruses probably spread along peripheral nerves. Sequential observation of viral antigen in spinal roots, dorsal root ganglia, and in autonomic plexi in the gut suggest terminal centrifugal spread from the CNS. Johnson and Johnson (9) also described neu-

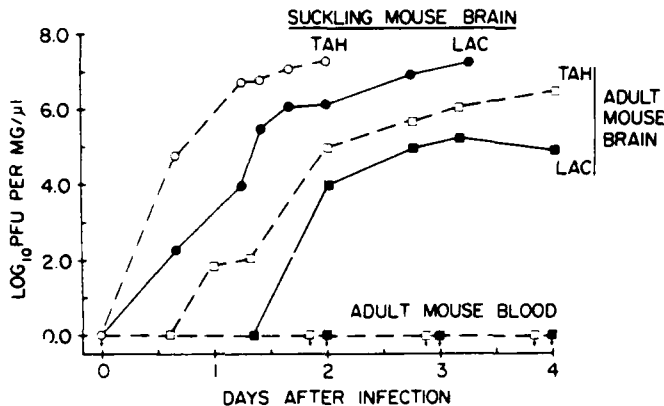


FIG. 3. Replication of LAC/original and TAH/181-57 viruses following ic injection of 700 pfu into suckling and adult CD-1 mice; both viruses kill mice by this route. Titers in plaque-forming units per milligram of tissue or per microliter of plasma. Each data point represents tissue pooled from three mice. ○ and ●, suckling mice; □ and ■, adult mice.

TABLE 3. VIRUS ANTIGEN IN THE TISSUES OF SUCKLING OR ADULT CD-1 MICE INFECTED SUBCUTANEOUSLY OR INTRACEREBRALLY WITH LAC/ORIGINAL OR TAH/181-57 VIRUSES AND SACRIFICED WHEN MORIBUND\*

Tissue	Suckling mice			Adult mice	
	LAC	TAH	2.8 log <sub>10</sub> , sc	LAC	TAH
	2.8 log <sub>10</sub> , sc	5.6 log <sub>10</sub> , sc		(2.8 log <sub>10</sub> , ic)	(2.8 log <sub>10</sub> , ic)
Brain					
Rhinencephalon	3	3	4	3	2
Cortex	3	3	4	3	3
Striatum	2	2	4	3	2
Hippocampus	4	2	3	4	4
Thalamus	3	2	4	2	3
Midbrain	3	3	3	2	3
Cerebellum					
Purkinje	4	1	4	2	3
Granule	2	-	3	-	+/-
Nuclei	3	2	4	3	3
Cord	4	2	3	ND	ND
Roots	1	-	1	ND	ND
Viscera					
Striated muscle	3	1	+/-	-	-
Heart	1	-	-	-	-
Other <sup>b</sup>	-	-	-	-	-

\* Suckling mice were moribund on days 3 to 4 (LAC (sc)), days 4 to 7 (TAH (sc)), or days 2 to 3 (TAH (ic)). Adult mice were moribund on days 4 to 5. ND, not done. Grading scale. 4, more than 75% of cells fluorescent; 3, 50 to 75%; 2, 25 to 50%; 1, 3 to 25%; +/-, less than 3%; -, 0%. Each time point represents the average of three animals.

<sup>b</sup> Other viscera: spleen, liver, gut, lung.

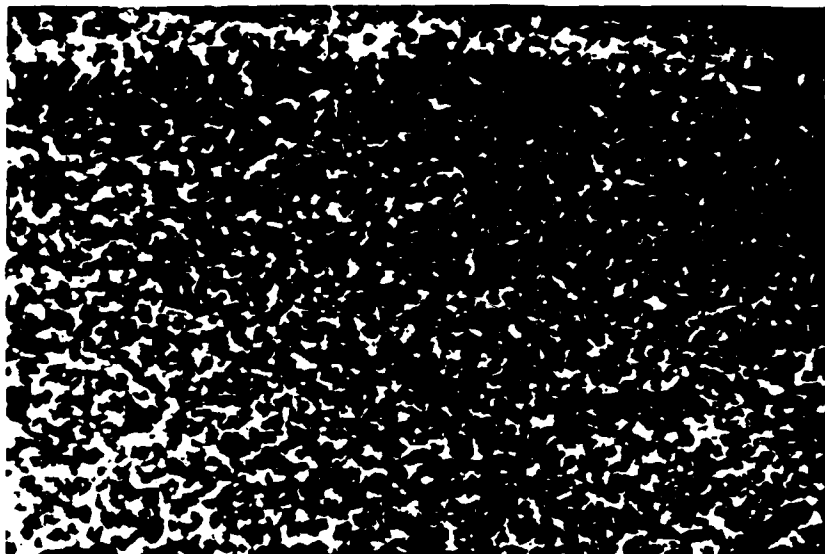
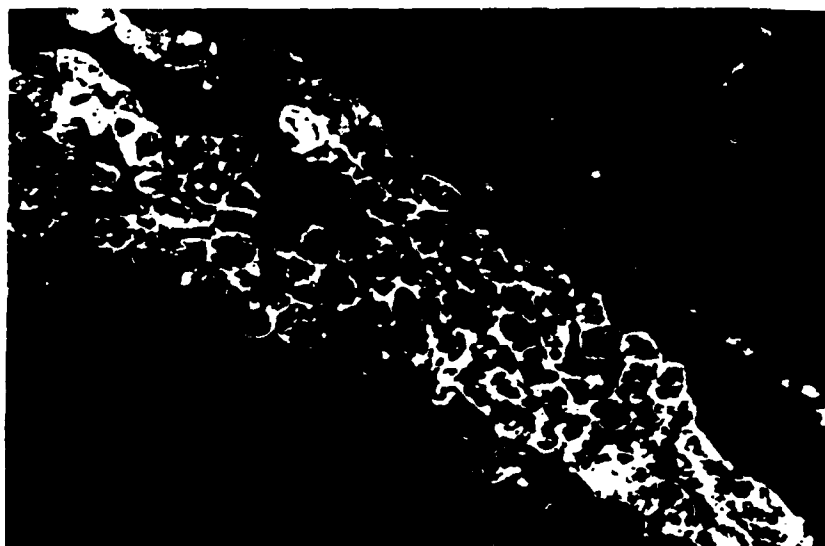


FIG. 4. Suckling mice, moribund after *sc* inoculation of 700 pfu of LAC/original virus. *Top*, Viral antigen in striated muscle. *Middle*, Viral antigen confluent throughout cerebral cortex. *Bottom*, Cerebral cortex with fields of necrosis (right). *Top*, Immunofluorescent stain;  $\times 500$ . *Middle*, Immunofluorescent stain;  $\times 200$ . *Bottom*, Hematoxylin-eosin and Luxol fast blue;  $\times 100$ .

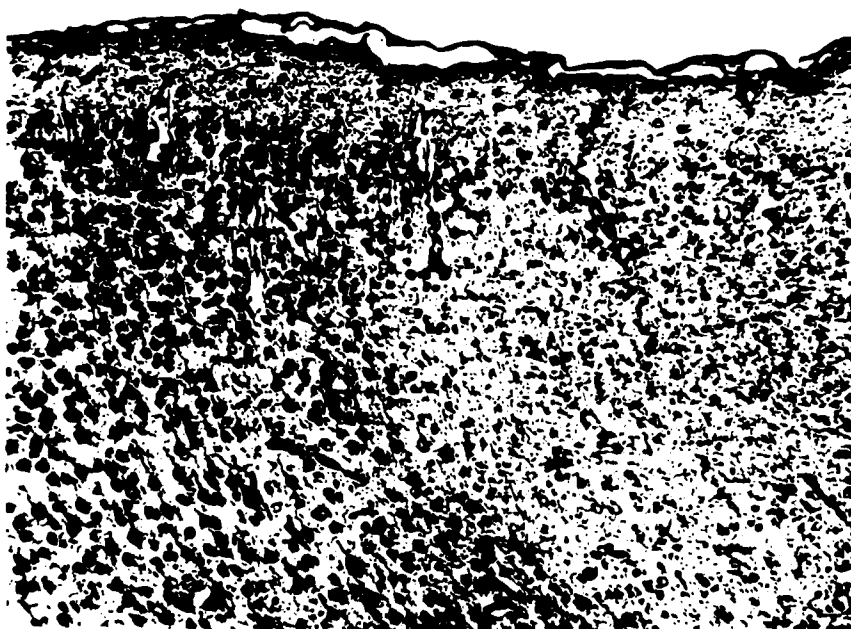
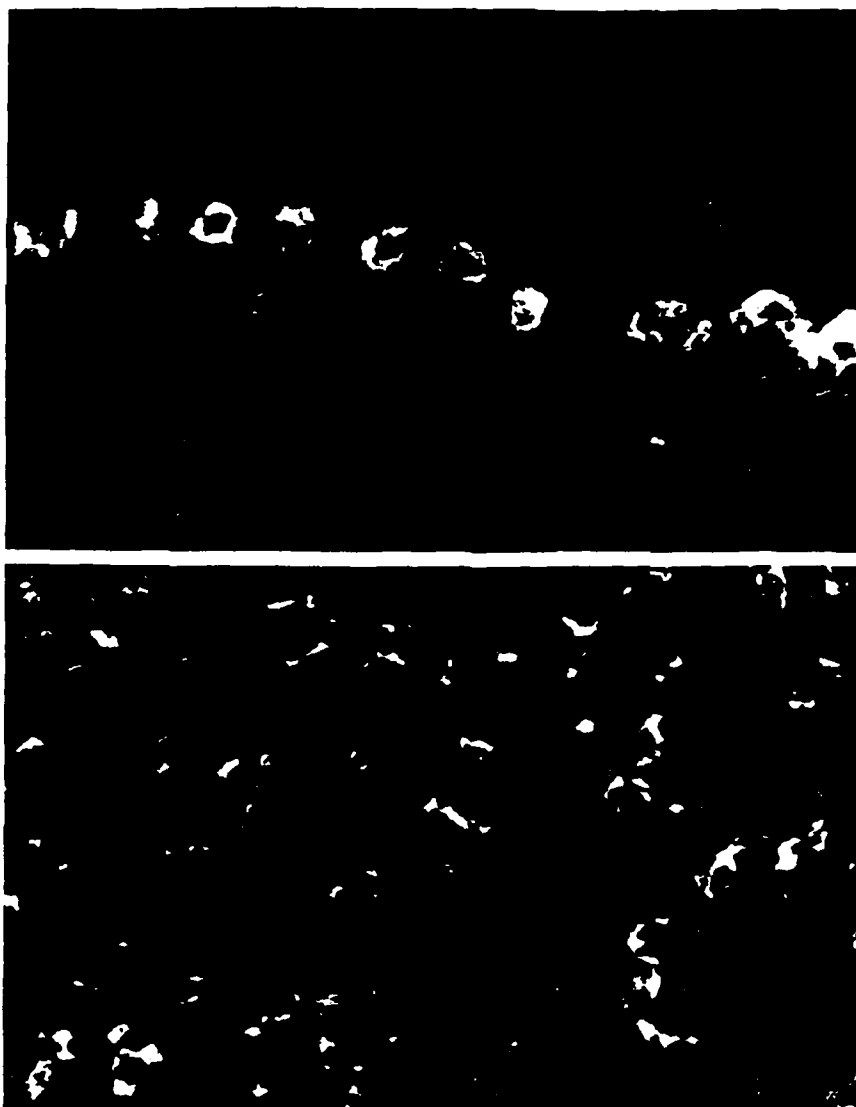


FIG. 5. Adult mouse, moribund after ic injection of 700 pfu of LAC/original virus. *Top*, Viral antigen localized to Purkinje cells of cerebellum. *Bottom*, Viral antigen in neurons of brain stem. Immunofluorescent stain;  $\times 500$ .



ronal spread, and Tignor and colleagues (21) suggested that, in addition to viremia, neuronal spread might play a role in CNS invasion.

#### COMPARISON OF VIRULENT AND AVIRULENT VIRUSES

The distinction between neuroinvasive LAC/original and noninvasive TAH/181-57 was clear and striking: virulence was associated with replication in striated muscle and production of a high titer viremia. These differences were accentuated by the use of a highly avirulent strain of Tahyna virus that permitted studies in suckling mice. The other, albeit more subtle, distinction between the two strains was the faster rate of replication within the CNS exhibited by TAH/181-57. This latter feature was also noted by Shope, Rozhon, and Bishop (16), using a non-neuroadapted strain of Tahyna virus.

Both of these characteristics of TAH/181-57 were undoubtedly associated with its history of more than 50 brain-to-brain passages. Malkova (12, 13) indicates that this strain resembled other Tahyna strains shortly after isolation and only acquired its low invasiveness after multiple brain passages.

The selection of viruses of high neuronotropism and low viremia potential by multiple CNS passages is a well-established observation in the early literature and was reported for poliovirus (the MV strain (15)), for yellow fever virus (French neurotropic strain (19)), for influenza (the neurotropic WS strain (18)), for lymphocytic choriomeningitis virus (Armstrong strain (3)), and for measles virus (hamster neurotropic strain (22)). The molecular mechanism underlying this affinity for neuronal cells and processes has never been elucidated, but genetic experiments with the bunyaviruses (see following data) suggest that it may be associated with fine differences between the viral receptors on neurons and other cells.

#### VIRAL GENES THAT DETERMINE NEUROINVASIVENESS

To investigate the viral genes associated with neuroinvasiveness, we have constructed reassortants between the two viruses compared in these studies and have avoided the use of mutagenized parents. Preliminary results (to be published elsewhere) indicate that virulence cosegregates with the M RNA and not with the S RNA; as yet, the genotype of the L RNA has not been

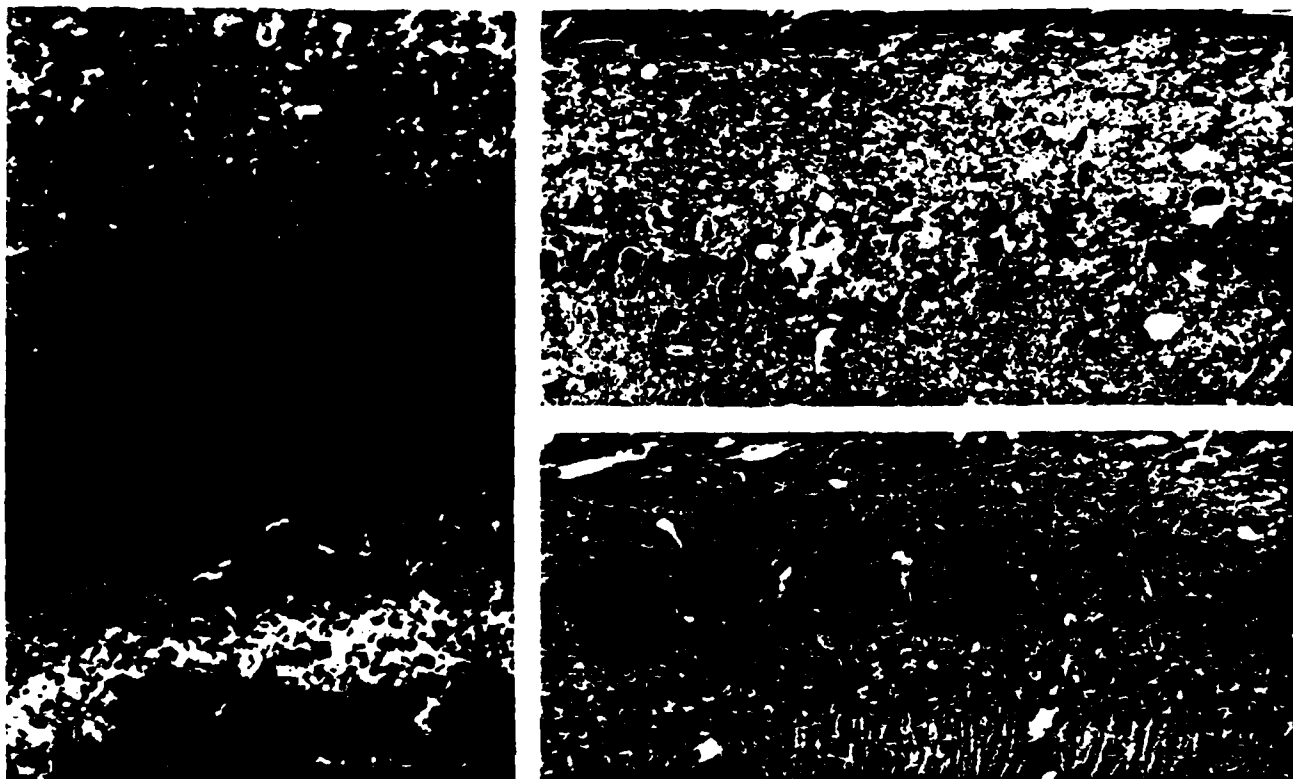


FIG. 6. Adult mice moribund after ic injection of 700 pfu of LAC/original virus. *Left*, Viral antigen in neurons of cerebral cortex, *above*, and the pyramidal cells of the hippocampus, *below*, with minimal antigen in the intervening corpus callosum. *Top right*, necrosis of

pyramidal cells of hippocampus. *Bottom right*, normal pyramidal cells in uninfected mouse. Figure 6 *left*, Immunofluorescent stain;  $\times 200$ . *Top right*, Hematoxylin eosin and Luxol fast blue;  $\times 300$ . *Bottom right*, Hematoxylin and Luxol fast blue;  $\times 300$ .

determined for these reassortants. These results are consistent with the work of Shope and Bishop (16), who studied reassortants between ts mutants of Tahyna and La Crosse viruses and also found that virulence cosegregated with the M RNA.

The M RNA encodes two virion structural proteins, the G1 and G2 envelope glycoproteins (2). Our observations with monoclonal antibodies are (7) that the G1 protein possesses a domain that is responsible for attachment to receptors on BHK cells and goose erythrocytes. We have also recently identified a fusion function (F. Gonzalez-Scarano, N. Pobjecky, and N. Nathanson, unpublished results) that is presumably associated with either G1 or G2 proteins. It may be speculated (see Shope *et al.* (16)) that the properties of low neuroinvasiveness and high neuronotropism are related to either attachment or fusion, the two critical early steps in viral infection. For instance, it is possible that the viral receptors on striated muscle cells and neurons differ in fine structure and that the G1 of LAC/original can bind efficiently to both receptors, whereas the G1 of TAH/181-57 binds avidly to neurons but inefficiently to striated muscle. Several studies to explore this hypothesis are now underway.

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Appendix 3

An Avirulent G1 Glycoprotein Variant of La Crosse Bunyavirus with  
Defective Fusion Function

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## ABSTRACT

La Crosse virus, a member of the California serogroup of the family Bunyaviridae, causes encephalitis in humans and in experimental rodents. A variant virus (V22) selected with a monoclonal antibody against the large (G1) glycoprotein showed diminished neuroinvasiveness after peripheral inoculation. This variant has an alteration in the fusion function, requiring a lower pH for activation of fusion, and demonstrating reduced efficiency of cell-to-cell fusion of BHK-21 cultures. V22 was studied in detail following infection by intraperitoneal or intracerebral routes in suckling, weanling, or adult CD-1 mice. It exhibited marked reduction in its ability to replicate in striated muscle and to produce a viremia; however, after intracerebral injection V22 virus replicated almost as rapidly in brain as parent La Crosse virus. V22 virus thus represents an example of reduced neuroinvasiveness associated with an alteration at a specific epitope of the G1 glycoprotein. This same epitope also influences the fusion activity of the glycoprotein.

## INTRODUCTION

The study of virus variants can provide a powerful approach to the delineation of the molecular basis of virus virulence. The ease of selection of antigenic variants has allowed investigation of the effect of alterations of virus functions on the tropism and virulence of different virus families. Several groups have shown that variants selected with monoclonal antibodies have reduced virulence. In the rabies system (2, 34) the genome of the variants with decreased pathogenicity has been sequenced (4), and as with other monoclonal variants (3), the alteration is a single nonredundant base substitution, leading to a single amino acid change. The biological function that is inhibited has not yet been studied. Several reovirus variants (31) selected with a single monoclonal antibody show a dramatic decrease in virulence when injected intracerebrally (ic) in suckling mice, and it has been postulated that this decrease in pathogenicity is due to altered receptor binding (32). We have selected a group of monoclonal antibody (10) variants of La Crosse (LAC) virus, and among them, a variant selected by one monoclonal antibody (807-22) showed decreased neuroinvasiveness, that is decreased ability to cause a central nervous system (CNS) infection after peripheral inoculation. Prototype variant 22 (V22) virus was also inefficient at mediating cell-to-cell fusion, either from within (FFWI) or without (FFWO). This report presents a comparison of V22 and parent LAC viruses with respect to fusion activity and pathogenesis in mice.



## METHODS

Monoclonal Antibodies The procedures for the generation of monoclonal antibodies against LAC virus have been described elsewhere (10). Briefly, weanling Balb/c mice were infected with approximately one LD-50 dose of LAC virus (LAC/original, passage 6) and the animals surviving three weeks later were re-challenged with LAC virus. The spleens were fused with mouse myeloma cells (19, 21) 2-4 days later and antibody secreting colonies selected with ELISA, then cloned in agarose. The protein specificity of the monoclonal antibodies was then determined by immunoprecipitation of solubilized, <sup>3</sup>H leucine labelled, LAC infected cell extract. All of the antibodies were directed against either the large (G1) glycoprotein or the nucleocapsid (N) protein (10, 13, 20, 27).

Selection of Variant 22 (V22) virus La Crosse/original (LAC) virus has been described (17). Ten-fold dilutions of LAC, in the form of a 10% suckling mouse brain suspension were combined with a low dilution (1:5 or 1:10) of 807-22 monoclonal ascitic fluid and held for 30 min at room temperature. The virus-antibody mixture was then inoculated on a monolayer of BHK-21 cells on 6-well plates and rocked gently for 60 min at room temperature, following which the inoculum was removed and the monolayer overlaid with Eagles Modified MEM containing 2% fetal calf serum (FCS) and a final concentration of 0.5% Agarose (Seaplaque, Marine Colloids Division, Rockland, Maine). Isolated plaques appearing at the end point of neutralization were picked and purified twice, using antibody during the initial stage of infection of each purification. The frequency of selection was calculated as (Titer of LAC Stock + Ascitic Fluid)/Titer of LAC Stock.

Variants were selected with monoclonal 807-22 on two occasions, one year apart. Unless otherwise specified, all of the studies noted here were done with a single clone termed V22. A second clone, V22F, selected independently, was used to confirm that these results apply to other variant viruses selected with this antibody and are not characteristic of a single spontaneous mutation. V22F was selected from a tissue culture stock of LAC, whereas V22 was selected from a 10% suspension of infected LAC suckling mouse brain homogenate.

Mice. Outbred albino Swiss mice (CD-1; Charles River Breeding Laboratories, Inc.) of various ages were used. All animal studies were done with single plaque-purified tissue culture stocks of LAC or V22. LD-50 titers were calculated by inoculating five weanling or adult mice or one litter of suckling mice with ten-fold dilutions of LAC or V22 viruses. For pathogenesis studies, suckling mice 48-72 hours old were inoculated intraperitoneally (ip) with 2200 pfu of either virus; weanling mice, 3 weeks old, were inoculated ip with  $10^5$  pfu and 8-10 week old adult mice were inoculated intracerebrally (ic) with 1000 pfu of either virus. Mice were infected with calibrated inocula of 0.05 or 0.02 ml.

Survival. Animals of each age group were infected and the survivors were counted every 8 hours for 15 days. Using 25-40 mice per group, mean survival times and standard deviations were computed.

Tissue titrations. The details of tissue titrations have been described previously (17). Mice were infected, sacrificed by decapitation, and bled into a tube containing 300 units of heparin. Selected organs (brain, quadriceps muscle, liver, and heart) were dissected from three mice and prepared as 10% suspensions in minimal essential medium (MEM) with 2% FCS. Samples were assayed by plaquing on BHK-21 cells and the titers were recorded as pfu per mg tissue or ul plasma; the assay had a sensitivity of 1 pfu per mg or per ul.

Plasma antibody response. The antibody response to inoculated virus was determined in the weanling mice used for tissue titration. Serial 2-fold dilutions of plasma were tested by ELISA using band purified virus as antigen, as described previously (12). Titers were recorded as the highest dilution of plasma which gave an OD reading at least twice the background reading of pre-infection plasma samples.

Immunofluorescent techniques. Immunofluorescent methods for observing viral antigen in frozen sections of infected mouse tissue have been described previously (17). The indirect immunofluorescent method was used with hyperimmune rabbit anti-LAC antiserum (neutralization titer 1:3200) in the first step, and a fluorescein isothiocyanate-conjugated goat antirabbit IgG (light and heavy chain specific, Cappel Laboratories, Inc.) in the second step. Both reagents were used at a dilution of about 1:16 and Evans Blue (final concentration, 0.5% v/v) was added to the conjugate as a counterstain. Brains were cut in parasagittal section and sections of torsos of suckling mice were cut in the transverse plane. Infection of skeletal muscle in suckling mice was evaluated by counting positive cells in three animals at each timepoint and determining an average number of infected cells. Controls for specificity were regularly included; the only nonspecific staining seen was confined to chondrocytes, cartilage, skin and intestinal mucosa.

Histology. Moribund mice were perfused, and the tissues stored in Bouin's solution, embedded in paraffin, and stained with Luxol fast blue and hematoxylin and eosin (17).

Plaquing of viruses The procedures used in determining plaque titers of LAC, V22, and V22F are described in detail elsewhere (17).

Cell-to-Cell Fusion Fusion from without (FFWO): The quantitation of FFWO using LAC virus has been described (11).

Fusion from within (FFWI): A subconfluent monolayer of BHK-21 (clone 13) cells on 96-well (Costar) plates ( $2.2 \times 10^4$  cells/well) was inoculated with LAC or V22 at an moi of 1.0, kept at room temperature for one hour, washed with PBS, then incubated with MEM with 2% FCS at 35°. Sixteen hours after infection the cells were washed with PBS, exposed to pre-warmed (37°) MEM with 10 mM Morpholinoethanesulfonic acid adjusted to the appropriate pH with NaOH, and incubated with MEM + 2% FCS at 37° for 30 min. The cells were then fixed and stained with Giemsa (Difco, Scientific Products). The nuclei and cells were then counted and the fusion index determined (33) as  $FI = 1 - (C/N)$ , where C is the number of cells and N the number of nuclei. Approximately 1700 nuclei were counted for each data point in the figures.

## RESULTS

### SELECTION AND CHARACTERIZATION OF V22 VIRUS

Antigenic variants were selected with antibody 807-22 at a frequency of  $10^{-5.8}$ , slightly higher than the average frequency for all LAC neutralizing monoclonal antibodies available in our laboratory (10). All of the antigenic variants obtained with our panel of monoclonal antibodies were screened for any alteration in virulence by inoculating 1000 pfu subcutaneously into suckling mice. The parent LAC virus and all other variants killed 100% of the inoculated mice at this dosage. V22, which killed about 20% of the animals, was then formally tested by determining the PFU/LD-50 ratio for suckling, weanling and adult CD-1 mice after intraperitoneal and intracerebral inoculations (Table 1). When compared with LAC virus in parallel experiments, there is a 100-fold to 1000-fold reduction in mortality following peripheral inoculation of V22 virus. The LD-50 after intracranial inoculation of V22 is not diminished consistently, indicating that the salient biological defect is reduced ability to penetrate the CNS. In spite of the low mortality following peripheral injection of V22, adults and weanling mice acquired an immunizing infection, since they survived a potentially lethal challenge with LAC given about three weeks after the initial infection with V22 and they developed antiviral antibody as measured in an ELISA.

V22 grew in BHK-21 cells at a rate equivalent to that of parent virus, but peak titers were usually  $0.5 \log_{10}$  lower than for LAC, regardless of the initial moi (Fig. 1A). When growth under agar was compared, the V22 plaques were noticeably smaller (Fig. 1B).

Since these data suggested that the deficiency responsible for decreased virulence was not poor interaction with cellular receptors, we looked at another major glycoprotein function. Cell-to-cell fusion has been used extensively for the demonstration of fusion activity in several virus families (1, 23, 33). We have recently shown that LAC virus is capable of fusing cells when absorbed at 4° and exposed to acidic pH (FFWO) (11). FFWI, that is fusion of cells that have been actively infected, also occurs with LAC virus, and the pH characteristics are similar to those of FFWO. In our assay, fusion of cells with LAC begins when the pH is dropped to 6.3, and is maximal, in the 30 min allotted, if the pH is below 6.0. V22 virus was able to mediate FFWI, but the pH of optimal fusion was lower, with fusion of cells noted only after the pH was dropped to 5.8; maximal fusion occurred at pH 5.0. The efficiency of fusion, even at this pH, was lower than the fusing efficiency of LAC (Fig. 2). FFWI at pH 3.8, 4.0, and 4.5 was also less efficient for V22, suggesting that the deficiency was not just due to an alteration of the optimal pH for fusion, but rather to reduced efficiency.

Because V22 grows to a lower titer than LAC in BHK-21 cells, in other experiments we compared the maximum fusion obtained with each virus, at the pH of peak fusing activity, regardless of the time of infection. For LAC, maximum fusion was obtained at 16 hours after infection at an moi of 1.0 PFU/cell. The fusion index, an index of the extent of cell fusion that takes into account both the number of cells fused and the size of the average polykaryon (33), was 0.95 (1.0 being the theoretical maximum, with an infinite number of nuclei in one giant polykaryon). For V22 virus, peak fusion was obtained at 17 hours, just before the cells begin to show extensive cytopathic effect, and the fusion index was 0.76. The pH requirements for FFWO were identical to those for FFWI for each LAC and V22.

A comparison of the amount of virus protein required for FFWO was then undertaken. The results (Fig. 3) show that greater amounts (4-fold at an index of 0.5) of V22 are required for FFWO and that the maximum level of fusion with the variant is lower than with LAC (0.65 vs 0.84).

To determine whether the V22 epitope was consistently associated with altered biological properties, an independently selected variant, designated V22F, was tested in mice. V22F resembled V22 virus in its reduced virulence (data not shown). The pH requirements for FFWI with V22F viurs were identical to those of V22 virus, and V22F also made small plaques under agarose.

#### PATHOGENESIS OF V22 VIRUS

Since viral replication and susceptibility to infection by bunyaviruses are age related (18), we sought to demonstrate the differences between LAC and V22 viruses by inoculating various age groups of mice by different routes.

To study differences in replication in peripheral tissues under conditions of uniform fatality, we inoculated suckling mice, a very permissive age group, with 2200 pfu intraperitoneally (ip). To accentuate differences in survival, three-week-old weanling mice were inoculated intraperitoneally with  $10^5$  pfu. To investigate replication isolated to the CNS, we inoculated eight to ten-week-old adult mice with 1000 pfu intracerebrally (ic), since virus cannot be isolated from non-neural tissues in this age group. We then contrasted the two viruses by comparing survival time, tissue titration, immunofluorescence, serological response, and brain histology.

Survival. Suckling mice inoculated ip with 2200 pfu of either virus died rapidly with a median survival time of  $3.3 \pm 0.3$  days (Fig. 4). The majority (85%) of weanling mice inoculated with 100,000 pfu ip of V22 survived infection, compared to a minority (35%) of the mice inoculated with LAC virus. Sick animals sat hunched, moved slowly, circled repetitively, were paretic, or had generalized seizures. Moribund animals exhibited irregular breathing, paralysis, and unresponsiveness. Adult mice inoculated with LAC virus died slightly more rapidly than mice inoculated with V22 virus, (mean survival times of  $5.0 \pm 0.3$  and  $6.0 \pm 0.3$  days, respectively).

Tissue titrations. To follow the course of infection, mice were sacrificed at regular intervals, and selected tissues were titrated for virus content. The data are presented in Figs. 5-7; the timepoints for asymptomatic mice represent pools of three animals, whereas sick or moribund groups represent pools of one, two, or three animals.

Replication in fatally infected suckling mice inoculated with 2200 pfu is shown in Fig. 5. Of particular note is the replication in peripheral sites. LAC virus was isolated in high titer from all of the extraneural tissues examined. However, V22 virus was only detected in peripheral tissues known to support LAC replication — skeletal muscle and heart — and then only in moribund animals. We were unable to isolate V22 from either plasma or liver at any time. Virus could be detected in the brains of animals inoculated with either strain within 24 hours after infection, and titers subsequently rose to high levels, although V22 lagged slightly behind LAC virus. Replication in the brain probably reflected seeding of the CNS, by either virus, during the initial passive viremia immediately following inoculation, thus eliminating the need for peripheral replication and explaining the similarity in growth curves in the CNS.



The replication of virus in weanling mice inoculated ip with 100,000 pfu is summarized in Fig. 6. Because the outcome varied, mice were sorted into three groups at the time of sacrifice; asymptomatic, sick, and moribund. Only the brain titers are plotted, since with the exception of traces of LAC virus from quadriceps, we were unable to grow either virus from plasma or from skeletal muscle. Comparing the two viruses, LAC was detected in the brain at two days but V22 was not isolated until 4 days after infection. Subsequently, LAC grew to higher titers in brain than did V22 virus. Among mice inoculated with each virus, there was a hierarchy of titers, moribund mice demonstrating the highest and asymptomatic mice the lowest levels.

To study differences in replication within the CNS, adult animals were inoculated ic with 1000 pfu (Fig. 7). The course of infection for both viruses was quite similar but LAC virus grew slightly better, with a peak brain titer of  $10^{5.0}$  pfu per mg compared to  $10^{4.5}$  pfu per mg for V22 virus. Thus V22 virus had only a slightly diminished ability to replicate in the CNS.

To determine whether V22 virus underwent phenotypic reversion during mouse passage, one isolate (Fig. 6, V22, day 5, moribund) was plaqued in the presence and absence of monoclonal antibody 807-22; the titers were identical. In addition, all V22 isolates continued to demonstrate the small plaque phenotype characteristic of V22 virus.

Immunofluorescent observations. The accumulation of viral antigen in peripheral and central tissues was followed with indirect immunofluorescence, in order to corroborate the tissue titration data, and to examine the CNS for differences in the localization of infection. The following summary is based on detailed tabulation of the observations. In suckling mice, cross-sections of head, thorax and abdomen were stained to document the decreased presence of V22 in skeletal muscle. Although V22 virus was able to replicate in skeletal muscle, V22 antigen-positive cells were less frequent and they were localized to foci in the paraspinal muscles in comparison to LAC virus which was widely distributed in muscle. An actual count of antigen-positive skeletal muscle cells, two days after infection, on 3 individual mice per virus, showed a mean of 345 for LAC virus and a mean of 48 for V22 virus-infected animals (data not shown). LAC antigen was also occasionally noted in heart muscle. In addition, the parasympathetic plexus of the gut and periaortic ganglia were consistently positive in animals infected with either virus. Skeletal muscle in weanling mice was consistently negative for antigen. No other peripheral tissues were examined.

The infected CNS of mice of each age demonstrated a very similar pattern regardless of the virus strain or the route of inoculation. In suckling mice, antigen first appeared at 48 hours in the striatum and rhinencephalon and by days 3 and 4 it was widespread throughout the neuraxis. All of the cells seemed to stain in the moribund suckling animals but there was sparing of the major white matter tracts. Weanling and adult mice demonstrated a more limited involvement of the brain, such that individual cells could be recognized. There were virtually no differences between moribund V22 and LAC virus-infected suckling or weanling animals. Adult mice demonstrated more LAC than V22 antigen, a finding that is consistent with the slightly higher titers reached by the parental strain (Fig. 7). V22 virus did not show altered tropism within the CNS; like LAC virus, it infected neuronal cells and spared the white matter.

Plasma antibody titers. Weanling mice were followed for plasma antibody responses by ELISA. Fig. 8 shows that infection initiated a brisk antibody response that was first evident at 4 days and rose to high levels by 13 days after infection. The response to both viruses was very similar.

Histological observations. Pathological examination was limited to the brains of moribund mice, since LAC virus does not cause extraneural lesions and since pre-moribund mice show few and variable CNS alterations.

As described previously (18), age has a major influence upon the brain lesions produced by LAC virus. Suckling mice exhibited an overwhelming neuronal necrosis, often accompanied by hemorrhages; the necrosis was predominantly seen in the cerebellum, cerebral cortex, olfactory bulb, and occasionally in the hippocampus or thalamus. There was little concomitant inflammation.

Weanling and adult mice had similar lesions, of two distinct types. Focal necrotic lesions of variable severity, accompanied by inflammation, were seen in the hippocampus and olfactory bulb. About one-half of the animals also showed striking, sharply demarcated focal areas of status spongiosus in the midbrain or pons and occasionally in the thalamus, and rhinencephalon.

Mice that were moribund after infection with V22 virus showed very similar lesions to those described above for parent LAC virus (Table 2). There were more lesions in the mice infected with LAC virus (52% had moderate or severe lesions) than in those infected with V22 virus (22% had moderate or severe lesions).

## DISCUSSION

Although the exact role of fusion in the biology of enveloped viruses is not well understood, there is general agreement that it is intimately involved with the early events in viral penetration (6, 14, 16, 22, 25). The envelope glycoproteins have been shown to mediate this function in several well characterized systems (1), and hydrophobic sequences have been identified as essential peptides for fusion (9, 28). In both the orthomyxoviridae and the paramyxoviridae a conformational change in the fusion protein is necessary for activation of fusion (15, 30).

The information currently available about the LAC virus glycoproteins is insufficient to predict which of the two glycoproteins contains the putative hydrophobic peptide that mediates this function. The complete sequence of the middle-sized RNA segment, which codes for the glycoproteins (8, 29) of the closely related Snowshoe Hare virus shows that this RNA could code for a hydrophobic sequence either at the carboxy terminus of one glycoprotein or the amino terminus of another, or within either of the two proteins (5). Using amino acid specific proteases that digest G1 when still attached to the viral membrane, we have shown that the exposure of virions to an acid environment leads to different cleavage patterns. Similarly, four of the G1 monoclonal antibodies previously generated, including 807-22, show diminished binding in an ELISA following acidification of LAC, and the pH of change correlates with the pH of activation of the fusion function (12). We interpret these findings as showing that the large glycoprotein G1 undergoes a conformational change at this pH. The identity of the actual fusion protein remains to be determined, since the conformational change elicited by acid may expose the small glycoprotein G2 or it may expose other sites on the G1 itself. Regardless, the altered fusion pH in a G1 monoclonal variant confirms that the large protein is intimately involved with this function.

We have demonstrated that a monoclonal variant deficient in the fusion function shows altered pathogenicity in mice. We had previously shown (17) that injection of mice with LAC virus by a peripheral (intraperitoneal or subcutaneous) route causes an infection characterized by plasma viremia secondary to replication in muscle. V22 virus was unable to replicate efficiently in muscle and it did not establish a viremia. Nevertheless, once the virus reached the CNS, it replicated with only slightly diminished efficiency in comparison with the parental strain. The distribution of antigen in the brain was quite similar to that of parent LAC virus and the pathological changes, though less severe than with the parent strain, were typical of an acute viral encephalitis. The antibody response was similar in both viruses. Thus we have established that V22 has diminished neuroinvasiveness with only minimal loss of neurovirulence. In this respect it resembles an attenuated strain of the related Tahyna virus, which also shows decreased replication in muscle.

Since the exact role of the fusion function in enveloped viruses is incompletely understood, we can only speculate on the relationship between altered fusion and reduced neuroinvasiveness. The altered fusion function could retard the initial steps in the infection of individual cells, particularly if endosomal vesicles in muscle cells have a higher average pH than cells in the CNS. Alternatively, local tissue conditions (acidic pH during infection, for example) may allow for direct fusion of viruses to the plasma membrane. The higher pH at which LAC fuses membranes (6.3) may be more easily attainable than the pH which activates the fusion function of V22 (5.8), accounting for a greater proportion of infected cells. It is interesting to note a similar correlation between fusion efficiency and peripheral virulence has been reported for mumps virus; neuroinvasive strains are better at mediating cell-to-cell fusion than non-neuroinvasive strains (26, 35).

Attenuated variants of other neurotropic viruses have been selected with monoclonal antibodies (7, 31, 32, 34). The attenuated variants of reovirus, rabiesvirus and coronavirus differ in several ways from the V22 variant. First, they exhibit reduced neurovirulence on direct intracerebral injection. Second, the mechanism of attenuation is different, since in the cases where it has been studied (31, 32), there appears to be altered receptor binding. Our studies establish that fusion, another important function of the external virion proteins, is also important in determining virulence.

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TABLE I

Virulence of LaCrosse (LAC) virus and of Variant 22 (V22) virus in CD-1 mice,  
expressed as log<sub>10</sub> pfu per LD-50\*

Route of injection	Virus	Age of Mice		
		Suckling	Weanling	Adult
Intracerebral	LAC	-0.3	0.3	0.52
	V22	0.9	1.77	1.97
Intraperitoneal	LAC	0.49	3.71	3.90
	V22	2.35	>6	>7

\*A tissue culture stock of each virus, with a titer of  $2 \times 10^8$  PFU/ml was used for all experiments. Swiss albino mice (CD-1), were inoculated with the appropriate volume (i.e. 0.02 ml, ip. 0.05 ml) of virus diluted in 0.75% bovine albumin/PBS. Five weanling or adult mice were inoculated per ten-fold dilution. Suckling mice experiments used one litter/dilution.

TABLE 2

Frequency of brain lesions in mice infected with La Crosse (LAC) or variant 22 (V22) viruses

Age of Mice	Virus	Dose (pfu) Route	<u>Proportion of Mice with Specified Lesions</u>			Moderate or Severe Lesions*
			Necrosis	Spongi-form	Inflam-mation	
Suckling	LAC	2,200	3/3	0/3	0/3	3/3
	V22	ip	1/3	1/3	0/3	1/3
Weanling	LAC	100,000	6/14	8/14	5/14	6/14
	V22	ip	4/10	3/10	6/10	2/10
Adult†	LAC	1,000	8/12	9/12	3/12	6/12
	V22	ic	5/10	4/10	2/10	2/10
Totals	LAC		17/29	17/29	8/29	15/29**
	V22		10/23	8/23	8/23	5/23**

\* Moderate and severe lesions: graded 3 or 4 on a scale of 1 to 4.

\*\*  $p = 0.044$  (significant by chi-square).

### LEGENDS

Figure 1 Comparative growth of LAC and V22 viruses in BHK cells. A) LAC or V22 was inoculated for 1 hour on BHK-21 cells at  $\text{moi}=1.0$  PFU/cell, and maintained at  $35^{\circ}$  in MEM supplemented with 2% FCS. At designated intervals, supernatant fluids were collected and frozen at  $-80^{\circ}$  until assay (see below). ● LAC; ▲ V22. B) LAC and V22 were plaqued on BHK-21 cells grown on 6-well plates by inoculating for one hour at room temperature, then overlaying, after removing the inoculum, with media (MEM+2% FCS) containing 0.5% agarose (Seakem HI, Marine Colloids). On the third day after infection, the cells were stained with 0.1% neutral red. For other experiments, not designed to compare plaque size, LAC plaques were read on day 2 and V22 plaques on day 3 after infection.

Figure 2 Fusion from Within by LAC and V22 viruses. A sub-confluent monolayer of BHK-21 cells on 96-well (COSTAR) plates ( $2.2 \times 10^4$  cells/well) was inoculated with LAC or V22 at  $\text{moi}=1.0$ , kept at room temperature for one hour, washed with PBS, then incubated with MEM + 2% FCS at  $35^{\circ}$ . Sixteen hours after infection the cells were washed with PBS, they were exposed to MEM at the appropriate pH, and incubated for 30 min. The cells were then fixed, stained and the fusion index ( $1-(\text{no. of cells}/\text{no. of nuclei})$ ) determined after counting the nuclei and cells (circa 1700 nuclei per data point). The 95% confidence limit for all points was  $\pm 0.05$ . ●: LAC; ▲: V22; ○ : uninfected control.

Figure 3 Comparison of fusion from without by LAC and V22 viruses. Virus was grown in BHK-21 cells and precipitated from clarified tissue culture supernatant with 7% polyethylene glycol 8000 in 362mM NaCl, banded in a 20-70% sucrose gradient in 1M NaCl, 10 mM Tris, pH 7.4. Concentrations of purified virus were measured after disruption in 0.1% Triton-X with the Bio-Rad method, using BSA as a standard. The virus was absorbed in a volume of 20 ul/Terasaki well for 1 hour at 4°, and the cells exposed to either pH 5.5 (LAC) or 5.0 (V22) buffer, neutralized, and incubated at 37° for 30 min. The 95% confidence limit for all points was  $\pm 0.05$  ● : LAC; ▲:V22.

Fig. 4. Survival of CD-1 mice inoculated with La Crosse/original (LAC) or variant 22 (V22) viruses. Each curve was based on a group of 25-40 mice.

Fig. 5. Replication of La Crosse/original (LAC) and variant 22 (V22) viruses in suckling CD-1 mice after intraperitoneal injection of 2200 pfu. Each point represents a pool of tissues from 3 mice.

Fig. 6. Replication of La Crosse/original (LAC) and variant 22 (V22) viruses in weanling CD-1 mice injected intraperitoneally with 100,000 pfu. Titers shown for brain only, since replication was negligible in extraneural tissues (see text).

Fig. 7. Replication of La Crosse/original (LAC) and variant 22 (V22) viruses in adult CD-1 mice after intracerebral injection of 1000 pfu.

Fig. 8. ELISA titers of plasma antibody against La Crosse virus in weanling CD-1 mice infected intraperitoneally with 2200 pfu. of La Crosse/original (LAC) or variant 22 (V22) viruses. Each point represents a pool of sera from 3 mice. Titers are expressed as the highest dilution which gave an OD reading at least twice background (see Methods).



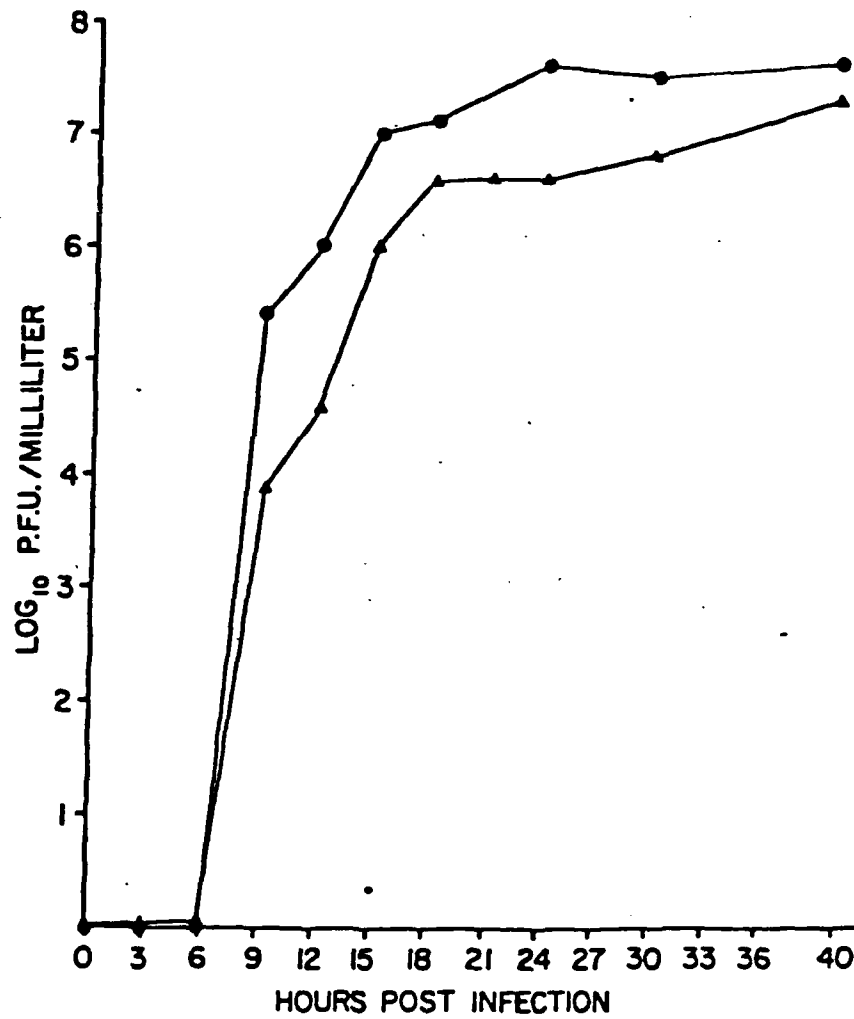


Fig. 1A

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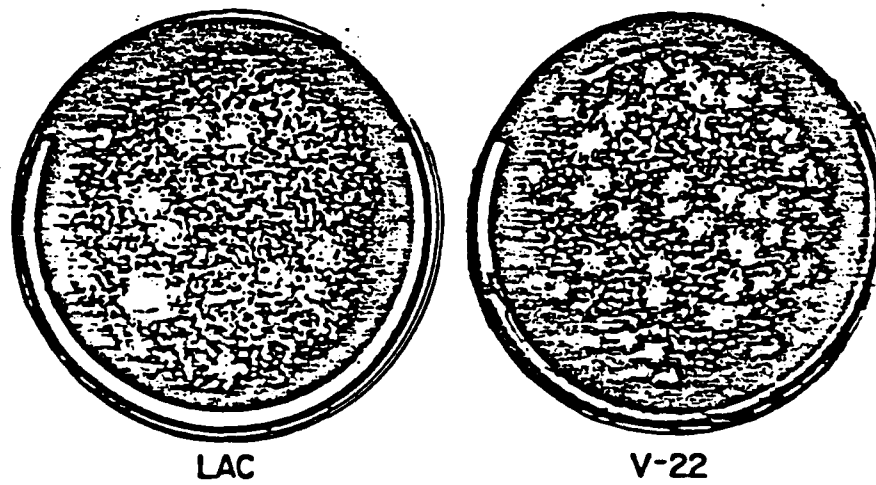


Fig. 1B

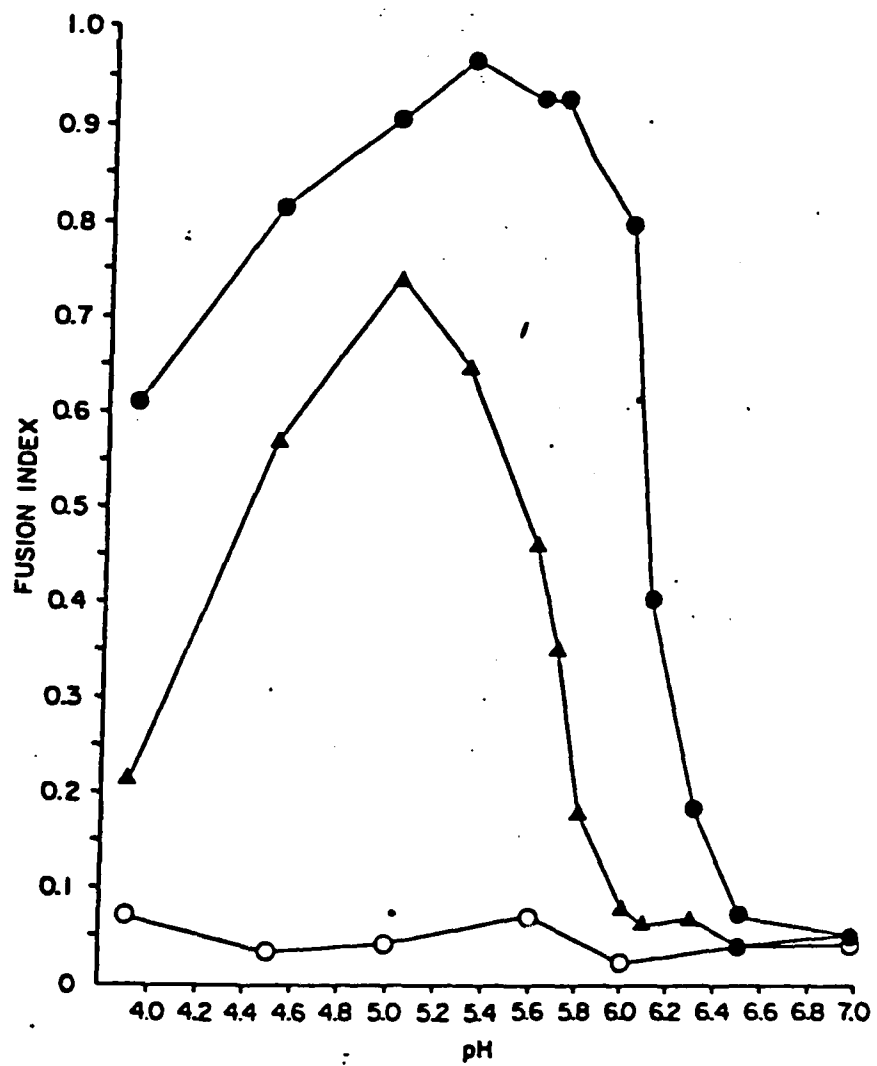


Fig. 2

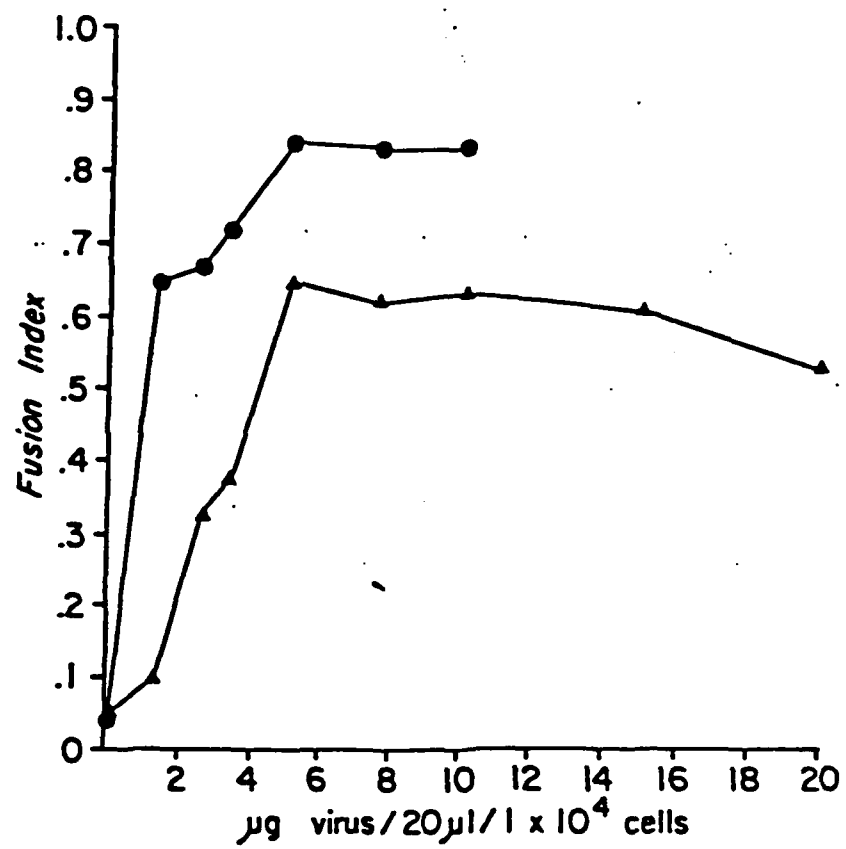


Fig. 3

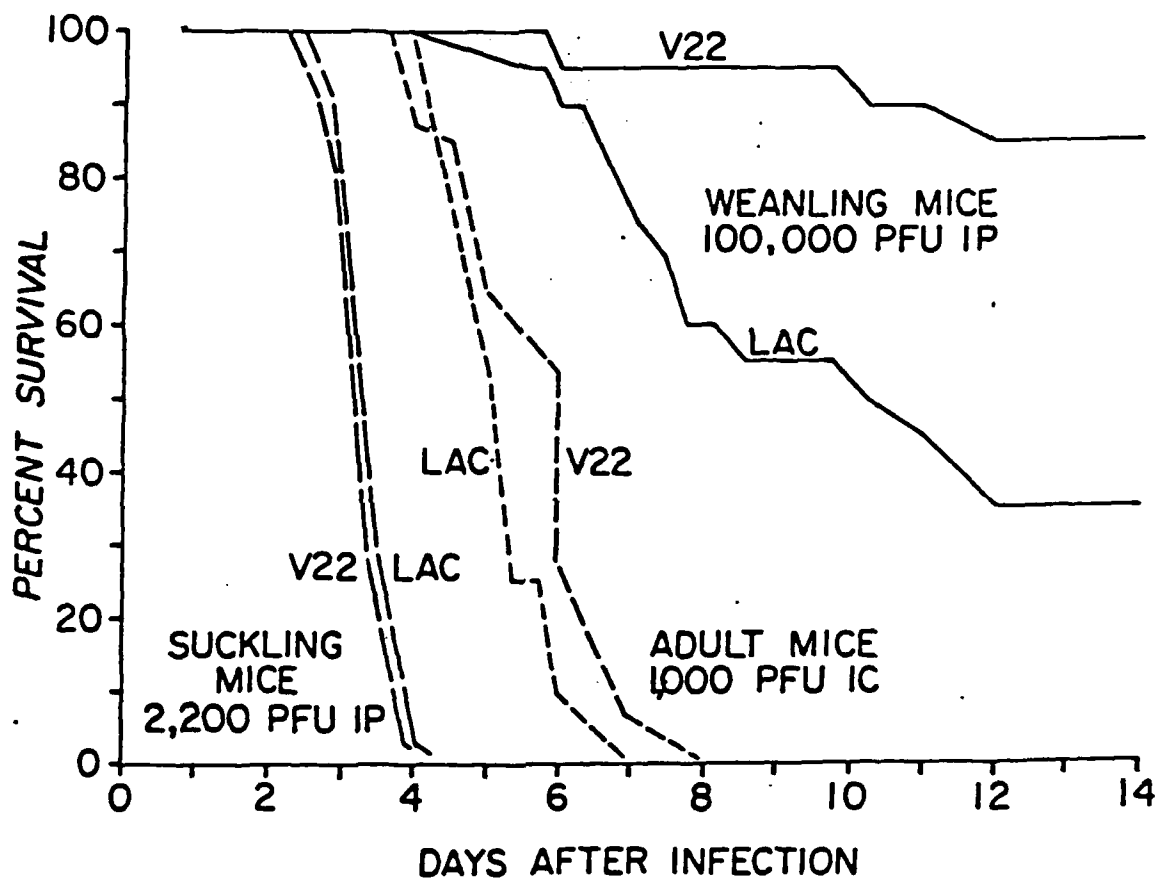


Fig. 4

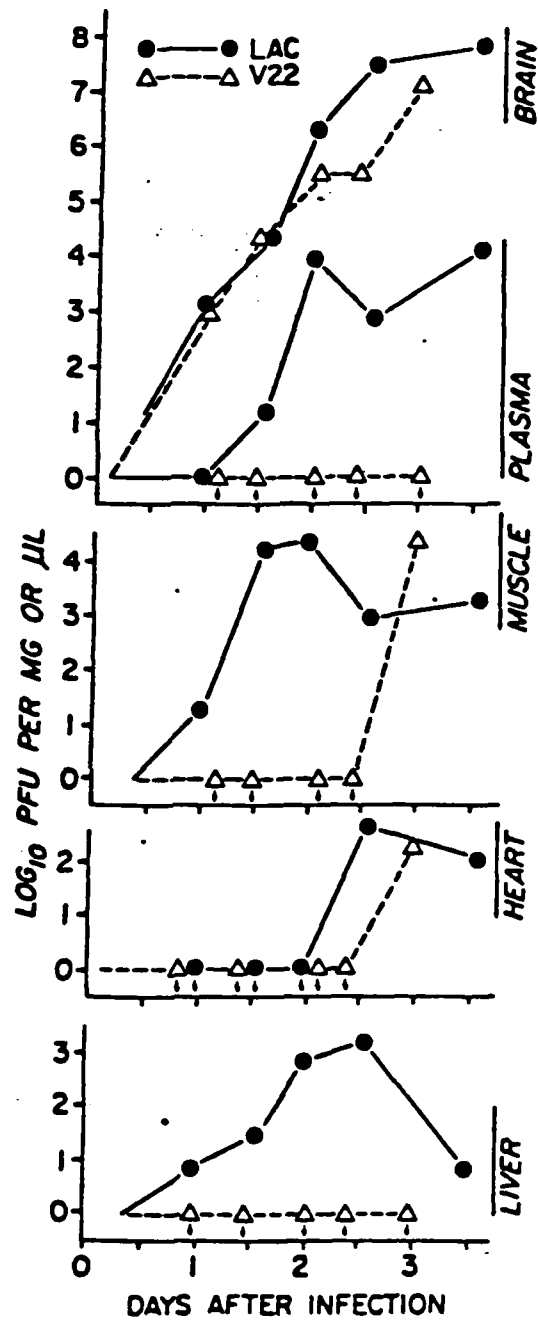


Fig. 5

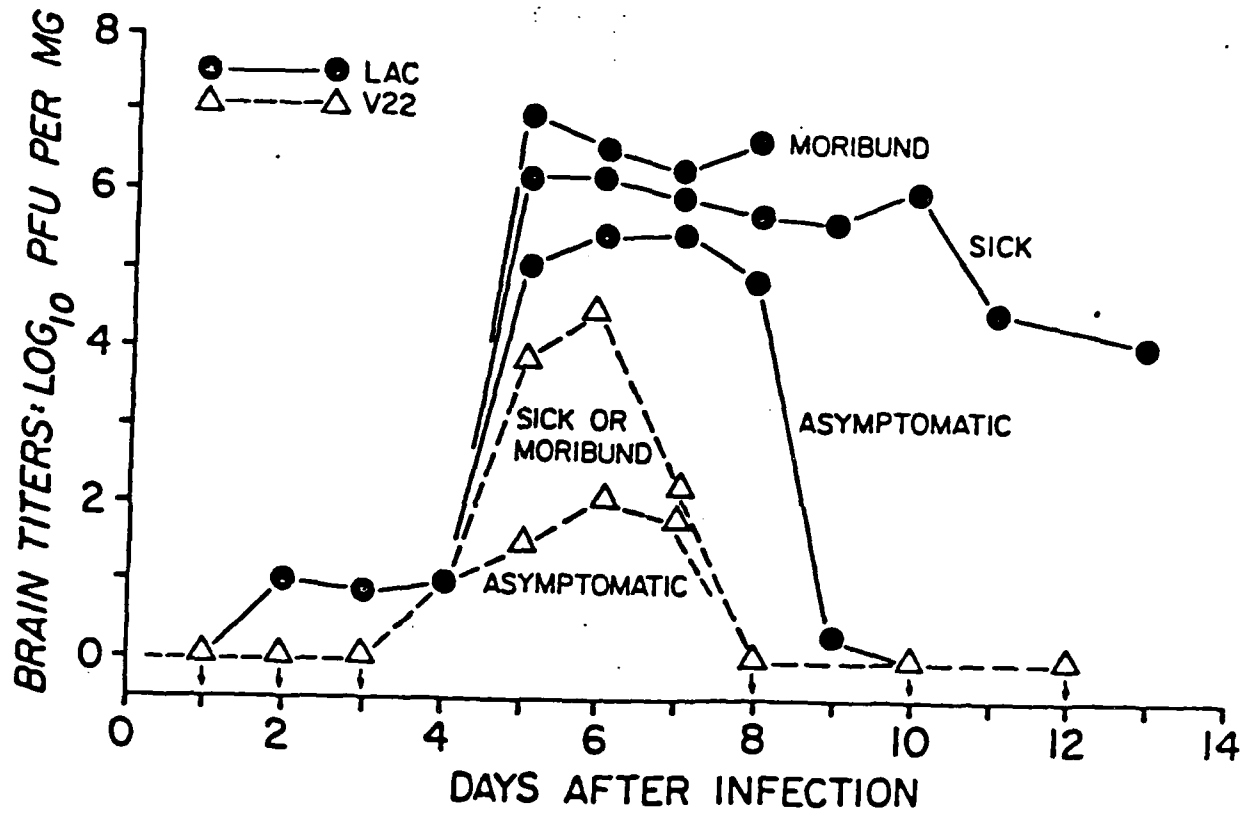


Fig. 6

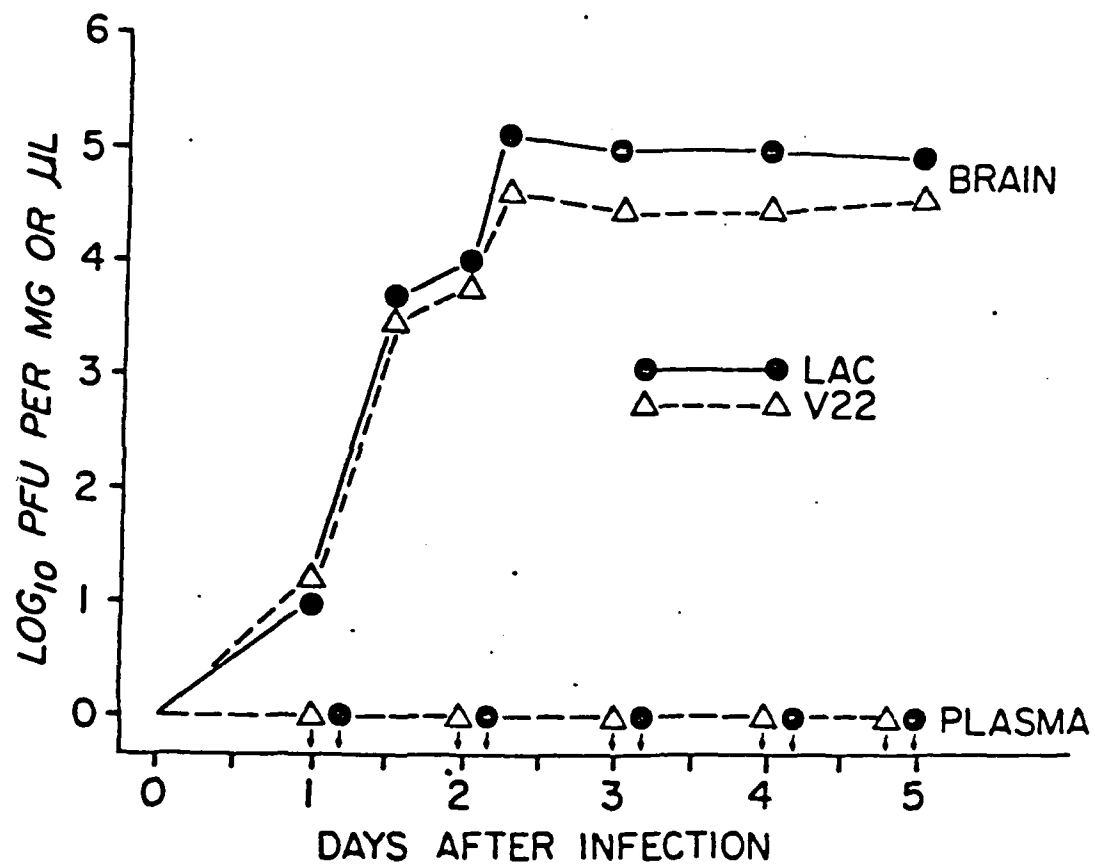


Fig. 7



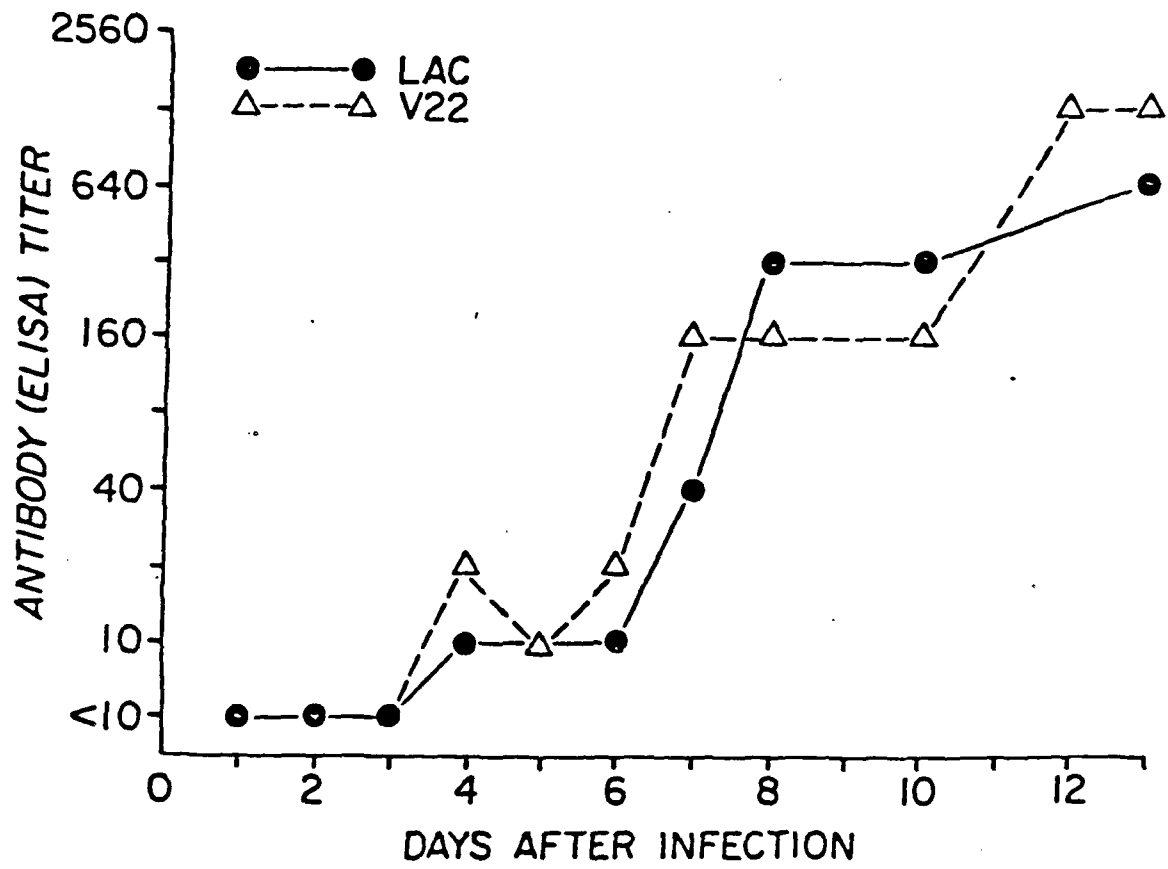


Fig. 8

## Appendix 4

VIROLOGY 140, 209-216 (1985)

### La Crosse Virus G1 Glycoprotein Undergoes a Conformational Change at the pH of Fusion

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La Crosse virus, a member of the family Bunyaviridae, can mediate cell-to-cell fusion after mild acidification. Either of its two envelope glycoproteins could potentially be responsible for this function. The large glycoprotein (G1) undergoes a conformational change at the pH of activation of the fusion function, resulting in both an alteration in the cleavage pattern produced by amino-acid-specific proteases, and in a change in its antigenicity, as defined by altered binding of monoclonal antibodies. © 1985 Academic Press, Inc.

#### INTRODUCTION

Like many other enveloped viruses, La Crosse virus (LAC), a member of the family Bunyaviridae, exhibits membrane-fusing properties when exposed to an acid environment (Gonzalez-Scarano *et al.*, 1984). To date, this fusion function has only been demonstrated by cell-to-cell fusion in tissue culture, either by absorption of virus followed by acidification (fusion from without—FFWO) or by acidification of cells undergoing a synchronized active infection (fusion from within—FFWI). The pH requirement using either method of cell fusion is identical (6.3) and is similar to that required by the alphaviruses and higher than the pH necessary for fusion with members of the family orthomyxoviridae (White *et al.*, 1981). In other respects (temperature, PFU/cell, extent of fusion) the fusion function of LAC resembles that of other systems where a pH drop is necessary for activation of fusion (Huang *et al.*, 1981; Mann *et al.*, 1983).

Although the exact role of fusion in virus-host interaction is controversial, and may vary for different families, it is widely accepted that fusion is important in the early events of viral penetration into the cell (Marsh and Helenius, 1980; Howe *et al.*, 1980). Fusion may occur at the cyto-

plasmic membrane, allowing the discharge of the virion contents into the cytosol, or it may take place following endocytosis of viruses into acidic prelysosomal or lysosomal vesicles (Lenard and Miller, 1982; Willingham and Pastan, 1980; Marsh *et al.*, 1983; Matlin *et al.*, 1982; Fan and Sefton, 1978), again resulting in the initiation of viral replication. In agreement with this concept, the fusion proteins are usually outer glycoproteins.

In two well-studied systems, the orthomyxoviridae and the paramyxoviridae, activation of the fusion function is accompanied by a conformational change of the fusion protein. In the orthomyxoviridae, activation is accomplished by acidification (Skehel *et al.*, 1982) and in the paramyxoviridae by cleavage of the F polypeptide into two fragments, F1 and F2 (Scheid and Choppin, 1977; Hsu *et al.*, 1981). In either case, the result appears to be exposure of hydrophobic residues at the NH<sub>2</sub> termini of F1 and HA2 (Skehel and Waterfield, 1974; Gething *et al.*, 1978; Wilson *et al.*, 1981). The actual fusion mechanism is poorly understood, but it can be inhibited, possibly by competition, by oligopeptides that mimic this region (Richardson and Choppin, 1983).

LAC virus has two envelope glycoproteins, G1 (120 kDa) and G2 (34 kDa)

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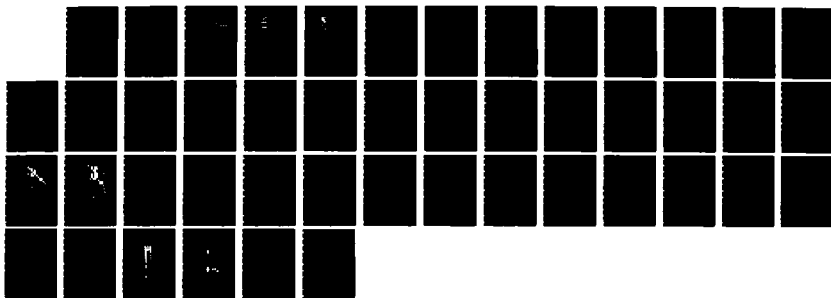
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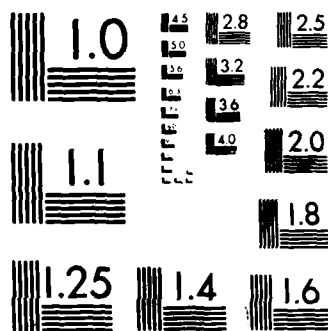
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## FRANCISCO GONZALEZ-SCARANO

which are present in approximately equimolar amounts (Obijeski *et al.*, 1976; Bishop and Shope, 1979). G1 contains the major antigen responsible for neutralization (Gonzalez-Scarano *et al.*, 1982) and is the putative attachment protein. There is no evidence that G2 is involved in neutralization and no viral functions have been attributed to it. In the absence of any sequence information about these two molecules, it is impossible to predict which is responsible for the fusion function. In this study we demonstrate that the G1 glycoprotein undergoes a conformational change at the pH of activation of the fusion function of LAC.

## METHODS AND MATERIALS

**Purification and 35S labeling of viruses.** LAC (La Crosse/original) virus was grown in BHK-21 cells using Eagle's MEM with Earle's salts supplemented with 10% fetal calf serum. The cells were infected at a multiplicity of infection of 0.01 for 1 hr at room temperature, then incubated at 35° in 5% CO<sub>2</sub>. The tissue culture supernatant was harvested when maximum cytopathic effect had occurred, clarified, and the virus precipitated overnight with 7% polyethylene glycol 8000 in 362 mM NaCl. The precipitate was banded in a 20–70% sucrose gradient in 1 M NaCl, 10 mM Tris-HCl, pH 7.4, pelleted, and resuspended in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4 (LSB). Protein concentrations were determined by absorbance at 280 nm, after determination of the extinction coefficient with the Bio-Rad method.

For preparing radiolabeled virus, BHK-21 cells were infected as above, but equal volumes of methionine-free media (Gibco) and Eagle's MEM were mixed and [<sup>35</sup>S]-methionine (New England Nuclear) added to a final concentration of 10–15 µCi/ml. A second 20–70% gradient was frequently run prior to final pelleting of labeled LAC.

**Monoclonal antibodies.** The production and characterization of monoclonal antibodies against LAC has been described previously (Gonzalez-Scarano *et al.*, 1982).

**ELISA assays.** Purified LAC was mixed with an equal volume of McIlvaine's ci-

trate-phosphate buffer, pH 5.8 or 7.3 and kept at room temperature for 15 min, following which it was neutralized and diluted to a concentration of 5.16 µg/ml with 50 mM NaHCO<sub>3</sub>, pH 9.5, and sonicated briefly in a Braun (Braun-Sonic 1510) sonicator.

Fifty microliters (258 ng)/well of antigen was then placed in plastic 96-well plates (Cooke, Micro ELISA) and held overnight at 4°. The plates were then rinsed with distilled H<sub>2</sub>O containing 0.005% Tween-80 and blocked for 30 min with ELISA buffer (0.5 M NaCl, 0.0005% Tween-80, 100 mM EDTA in phosphate buffer, pH 7.4) containing 10% agamma horse serum. The test antibody was diluted in ELISA buffer containing 4% serum added to appropriate wells, incubated for 1 hr at room temperature and washed, following which peroxidase-conjugated rabbit anti-mouse Ig (light and heavy chain specific, Cappel Laboratories) was added and held for 30 min at room temperature. The substrate (0.03% tetramethylbenzidine in 0.1 M sodium citrate buffer, pH 4.5, with 0.012% H<sub>2</sub>O<sub>2</sub>) was added and the optical density at 620 nm was read in a Titertek Multiskan MC. The average value for quadruplicate identical wells is reported after subtraction of background optical density, measured using ascites fluid obtained from Pristane-primed mice injected with Sp2/o myeloma cells.

**Protease cleavage.** Bovine pancreatic trypsin (TPCK-treated), fibrinolysin from porcine blood, elastase, and thermolysin were obtained from Sigma. Chymotrypsin was obtained from Calbiochem and staphylococcal protease V8 from Miles Scientific. Trypsin reactions were carried out in 100 mM Tris-HCl, pH 7.0, 10 mM CaCl<sub>2</sub>, 60 mM NaCl with a final concentration of enzyme of 100 µg/ml and stopped with bovine or soybean trypsin inhibitor (final concentration 200 µg/ml) or by boiling for 2 min in Laemmli's sample buffer (Laemmli, 1970). Other reactions were carried out in 100 mM Tris, pH 8.0, with a final enzyme concentration of 100 µg/ml and stopped by boiling in Laemmli's sample buffer.

## LA CROSSE VIRUS G1 GLYCOPROTEIN

Virus was treated with citrate-phosphate buffer, pH 5.8 or 7.3 for 15 min at room temperature, then neutralized with Tris-HCl prior to protease cleavage.

**SDS-PAGE.** Polyacrylamide gel electrophoresis in 12.5% resolving gels was done with the Laemmli system (Laemmli, 1970). The gels were fluorographed with Enhance (New England Nuclear).

## RESULTS

*Acidification Results in Altered Cleavage by Trypsin*

We consistently found that the large glycoprotein, G1, is the structural protein most susceptible to cleavage with common proteases following digestion of purified whole virions. The internal proteins, L and N were relatively resistant to cleavage under these circumstances, and the small glycoprotein, G2, was variably susceptible, depending on the conditions and the protease. These findings are illustrated in Fig. 1.

At room temperature, with trypsin as the protease, digestion of virions maintained at neutral pH results in the removal of the G1 glycoprotein and the appearance of one major product of 100 kDa, and a degradation product of less than 15 kDa (Fig. 2, lane 3). When the reaction is carried out with virions that have been mildly acidified to pH 5.6 with citrate phosphate buffer and then neutralized, the major product is a 72-kDa peptide (Fig. 2, lane 2). Using a series of buffers in the pH range of 6.0-6.8 for acidification of virus prior to cleavage (Fig. 3, lanes 2-7), we noted that the smaller, 72-kDa peptide appeared only when buffers with pH 6.4 or below were used for acidification of virus. Thus, there is a good correlation between the pH of activation of fusion, which is 6.3, and the pH at which the 72-kDa peptide appears (Gonzalez-Scarano *et al.*, 1984).

Labeled trypsinized virus was then pelleted through a cushion of 25% sucrose and the pellet analyzed with SDS-PAGE. Both the 100-kDa product of neutral trypsinization and the 72-kDa product of cleavage after acidification remain at-

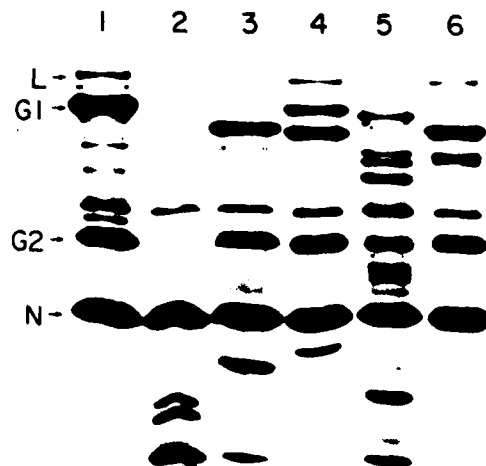


FIG. 1. G1 protein is most susceptible to protease cleavage. Band-purified [ $^{35}$ S]methionine-labeled LAC was suspended in 100 mM Tris-HCl, pH 8.0, and treated with proteinase (100  $\mu$ g/ml) at the indicated temperature for 30 min. The reactions were stopped by boiling in Laemmli's sample buffer and run on 12.5% SDS-PAGE with Tris-glycine buffer (Laemmli, 1970). Lane 1, LAC, no protease; lane 2, chymotrypsin, 37°; lane 3, elastase, 23°; lane 4, fibrinolysin, 37°; lane 5, staphylococcal protease V8, 37°; lane 6, thermolysin, 37°.

tached to the virion (data not shown). The low-molecular-weight degradation products could not be recovered in the pellet.

Previous investigations had shown the appearance of two digestion products of G1, 95 and 67 kDa (Kingsford, 1981) when trypsin cleavage (10  $\mu$ g/ml) was done at 35° under neutral pH. When we digested virions at 37° and neutral pH, we noted that bands corresponding to the 100-kDa and the 72-kDa peptides were seen and in addition another band was noted running at 61 kDa (Fig. 4, lane 2). However, the higher-molecular-weight species predominated. After acidification to pH 5.6 followed by neutralization and trypsin cleavage at 37°, the 72-kDa band is the major degradation product of G1 (Fig. 4, lane 3). Thus, acidification leads to different trypsinization patterns for G1 whether the reactions are carried out at 23° or 37°, but the differences are more marked at the lower temperature.

FRANCISCO GONZALEZ-SCARANO

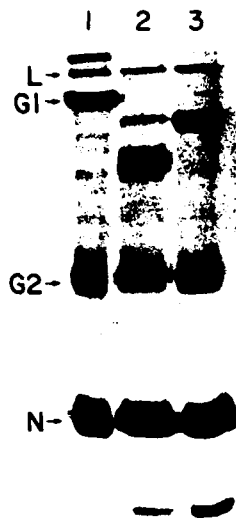


FIG. 2. Acidification results in different products of trypsin cleavage. [<sup>35</sup>S]Methionine-labeled LAC was treated with either acetate buffer (100 mM, pH 5.6) or citrate-phosphate buffer, pH 7.3 for 15 min at room temperature, then neutralized with 100 mM Tris-HCl, pH 7.0, and digested with 100  $\mu$ g/ml trypsin in a reaction mixture containing 60 mM NaCl and 10 mM CaCl<sub>2</sub>. After 20 min at room temperature the reaction was stopped by the addition of 200  $\mu$ g/ml soybean trypsin inhibitor and boiled for 2 min in Laemmli's sample buffer. The samples were then run on a 12.5% acrylamide gel. Lane 1, acidified, uncleaved LAC; lane 2, pH 5.6 treated trypsinized LAC; lane 3, pH 7.3-treated trypsinized LAC.

Similar findings, that is an alteration of the cleavage pattern when virus had been acidified, were seen with other proteases—elastase, fibrinolysin, chymotrypsin, and staphylococcal protease V8, although the number and molecular weights of the degradation products varied widely (data not shown).

The small glycoprotein, G2, was also noted to be partially cleaved with trypsin

and fibrinolysin, although clear-cut cleavage products were not identified. This was more prominent with reactions carried out at 37° than with reactions done at room temperature. The data in Fig. 5A, with the accompanying densitometer pattern, show that there is diminution in the G2 signal after cleavage with fibrinolysin at both pH 5.8 and 7.3, but that the diminution is more marked after acidification. Panel B is a time course of fibrinolysin treatment, and it shows that the G2 signal is diminished only after G1 cleavage is extensive. The N protein was not digested significantly under these con-

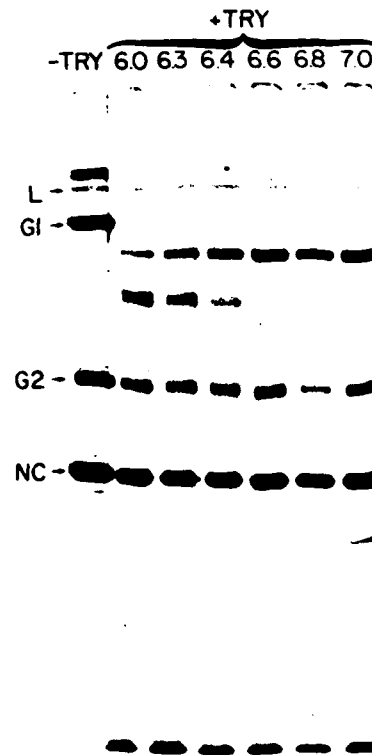


FIG. 3. The change in cleavage pattern after trypsin treatment correlates with the pH of activation of fusion function. [<sup>35</sup>S]Methionine-labeled LAC was treated with citrate-phosphate buffer at the indicated pH for 15 min at room temperature, then with 100 mM Tris-HCl to neutrality, and digested with trypsin as in the legend to Fig. 1. The reaction was stopped with bovine trypsin inhibitor, Laemmli's sample buffer was added, and the samples were boiled and run on a 12.5% acrylamide gel.

# LA CROSSE VIRUS G1 GLYCOPROTEIN

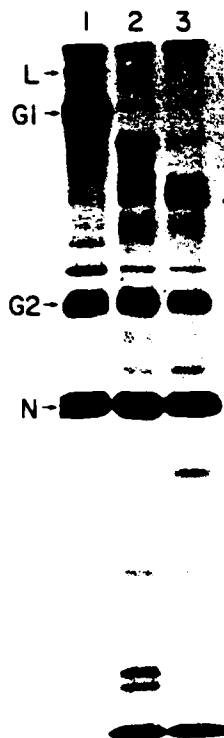


FIG. 4. Trypsin cleavage at 37°. <sup>35</sup>S-labeled LAC was acidified with citrate-phosphate buffer, pH 5.6 or 7.3, held at room temperature for 15 min then cleaved with trypsin as in the legend to Fig. 1, except that the reaction was held for 90 min at 37°. Lane 1 acidified, uncleaved LAC; lane 2, pH 7.3 trypsinized LAC; lane 3, pH 5.6 trypsinized LAC.

ditions. While this was shown primarily with fibrinolysin, close inspection of Fig. 4 reveals that the G2 signal is diminished at pH 5.6 when cleavage with trypsin is performed at 37°, rather than at room temperature. The higher temperature may be necessary for this effect to be noticeable.

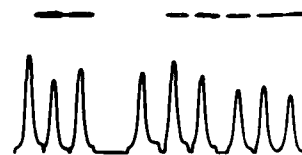
## Monoclonal Antibodies Demonstrate Reduced Binding to Acidified Virus

A number of monoclonal antibodies directed against G1 have been used for the characterization of this molecule. Among those tested (Gonzalez-Scarano *et al.*, 1982) four antibodies showed diminution in

binding, ranging from 32 to 72% of control, to virus that had been acidified prior to use as antigen in an ELISA assay. The antibodies that demonstrate this effect are a distinct cluster of epitopes (Najjar *et al.*, in preparation) on the G1 molecule. Two of the antibodies are able to inhibit LAC-mediated hemagglutination of goose RBCs yet are incapable of neutralizing the virus, although most neutralizing antibodies also inhibit hemagglutination (Gonzalez-Scarano *et al.*, 1982; Kingsford *et al.*, 1983). One of the other antibodies neutralizes related California serogroup viruses but not LAC, and antibody 807-22 both neutralizes and inhibits hemagglutination. Figure 6 shows the binding



FIG. 5. Fibrinolysin cleavage of LAC. [<sup>35</sup>S]Methionine-labeled LAC was treated with citrate-phosphate buffer, pH 5.8 or 7.3, neutralized with 100 mM Tris-HCl, and cleaved with fibrinolysin, 100 µg/ml at 37° for the period of time indicated. (A) Lane 1, LAC, uncleaved; lane 2, pH 5.8, 30 min cleavage; lane 3, pH 7.3, 30 min cleavage. (B) Lane 1, uncleaved; lane 2, pH 5.8, 2.5 min digestion; lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 40 min. Densitometer scan (Zeihn-LKB) of G2 signal.





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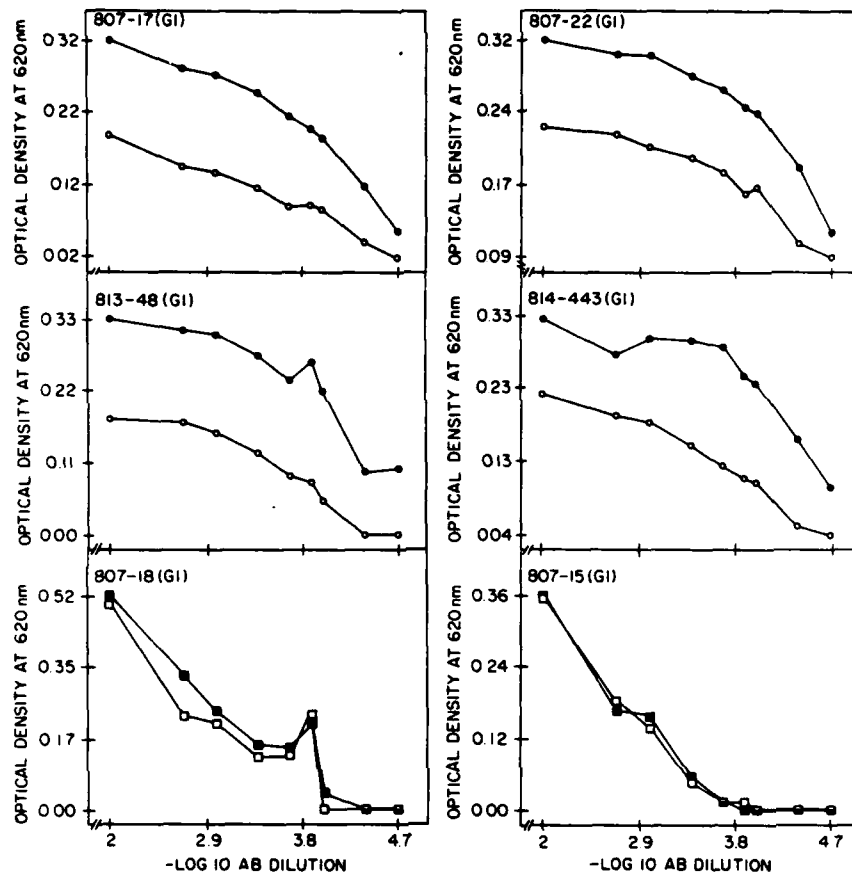


FIG. 6. Monoclonal antibodies show reduced binding to acidified virus. Purified LAC was acidified with citrate-phosphate buffer, neutralized, sonicated, and placed in ELISA plates at 258 ng/well, and specific binding of G1 monoclonal antibodies was determined for pH 5.8 or 7.3 treated virions. Circles: Monoclonal antibodies that show reduced binding to acid treated virus. ○ pH 5.8, ● pH 7.3. Squares: Representative G1 monoclonal antibodies that bind equally to both acidified and neutral virus, □ pH 5.8, ■ pH 7.3. All monoclonal antibodies directed against the nucleocapsid protein showed no diminution in binding at pH 5.8.

curves for the four antibodies that demonstrate reduced binding to acidified virus as well as for two antibodies that bind equally well to acidified and neutral virus. These last two antibodies (807-18 and 807-15) are capable of neutralization and inhibition of hemagglutination and their binding curves are representative of those of most of our monoclonal antibodies that are directed against LAC G1.

One antibody, 807-22, neutralized LAC to an extent sufficient to select monoclonal antibody virus variants. One variant selected with this antibody has been studied

extensively (Gonzalez-Scarano *et al.*, submitted) and shows lowering of the pH requirement for the activation of fusion.

#### DISCUSSION

These results show that the G1 protein of LAC undergoes a conformational change at the pH of activation of its fusion function. Together with data presented elsewhere (Gonzalez-Scarano *et al.*, submitted), which show that a G1 monoclonal variant has a markedly different pH requirement for activation of fusion,

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this study demonstrates that the large glycoprotein has a crucial role in the initial steps of fusion by LAC. In addition, the data demonstrate that the small glycoprotein G2 becomes more susceptible to protease cleavage at this pH. The fact that the changes in cleavage sites persist, in spite of neutralization of the virus after acidification, shows that the conformational changes are irreversible once established (Skehel *et al.*, 1982).

The decrease in binding of G1 monoclonal antibodies to virus that had been acidified confirms the presence of a conformational change, this time by alteration in antigenicity. Again, as in the protease experiments, the changes persisted after neutralization of the acidified virus. The group of monoclonal antibodies that show this phenomenon define a distinct cluster of epitopes on the G1 molecule, since the binding of most of the G1 antibodies to acidified virus was unchanged. Functionally, these antibodies are harder to characterize: two of them neutralize (one of them, 813-48 does not neutralize LAC, but neutralizes other members of the California serogroup). Antibody 807-22, the only one of these with a high neutralizing titer against LAC, selected variants that demonstrate different pH requirements for the activation of fusion, confirming that this antibody is directed against a part of the molecule that is intimately involved with this function.

The data for the H3 hemagglutinin of influenza (Webster *et al.*, 1983) also show alteration in specific sites following acidification of virus. Since the three-dimensional structure of this hemagglutinin has been elucidated, the epitopes affected can be mapped on the molecule (Wiley *et al.*, 1981). Similarly, Yewdell *et al.* (1983) found that only certain sites on the H1 subtype hemagglutinin were affected by acidification, although there were differences with the findings reported with the H3 subtype. They concluded that the antigenic changes noted after acidification were incidental to the conformational change accompanying fusion, since in all likelihood both hemagglutinin subtypes utilize similar mechanisms for the activation of fu-

sion. Our data lead to a different interpretation for LAC, since antibody 807-22 defines both a pH-sensitive epitope and a functionally changed variant.

This study does not identify the protein containing the hydrophobic residues that are presumed to be involved in fusion, but one can speculate on the role of the two proteins based on the information available. The large glycoprotein contains the site of attachment to cellular receptors, a role extrapolated from its involvement in neutralization and inhibition of hemagglutination, and it is highly antigenic. Based on its mobility in reducing and nonreducing gels, it appears to have few, if any, intramolecular disulfide bonds. G2, on the other hand, is a poor antigen and its mobility on SDS-PAGE changes significantly after reduction (data not shown). It may provide structure to a glycoprotein complex and its low antigenicity and protease resistance suggest a protected location. Although G2 becomes susceptible to cleavage after acidification, this susceptibility is not evident until after G1 has been cleaved. At present, it is unclear whether the conformational change undergone by G1 is intended to expose G2, or to expose other sites on the G1 molecule. The elucidation of these questions will have to be undertaken with purified protein preparations, or analysis of deduced sequences after cloning cDNA of the RNA segment coding for these proteins.

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Appendix 5

12-19-84

Epitopes of the G1 Glycoprotein of La Crosse Virus  
Using Variant Viruses Selected with Monoclonal Antibodies

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Abstract

Antigenic sites on the G1 glycoprotein of La Crosse bunyavirus were defined by constructing a panel of neutralizing and non-neutralizing monoclonal antibodies (Gonzalez-Scarano et al., 1982). To analyze the relationship between the individual epitopes delineated by monoclonal antibodies, 11 neutralizing antibodies were used to select variant viruses. These variant viruses were tested against the panel of anti-G1 protein monoclonal antibodies by neutralization and by ELISA. The neutralization tests assigned the 11 epitopes to 5 groups, consisting of 6, 2, 1, 1, and 1 epitopes. ELISA tests gave a similar pattern, but also demonstrated interrelationships between 4 of the 5 epitope groups, suggesting that there may be a single immunodominant antigenic site on the G1 protein. When 8 non-neutralizing anti-G1 monoclonal antibodies were tested in ELISA, they fell into 3 of the 5 epitope groups defined by neutralization; there was no evidence of a separate non-neutralizing antigenic site on the G1 protein.

## INTRODUCTION

Since the discovery of techniques for the preparation of monoclonal antibodies (Kohler and Milstein, 1975), a variety of methods have been used to delineate the antigenic sites on viral molecules that can elicit neutralizing antibodies (Gerhard et al., 1981, Yewdell and Gerhard, 1981). Because such antibodies are frequently capable of protecting the experimental animal from infection, their study is of practical as well as theoretical interest.

In general, neutralizing antibodies interfere with events that occur early in the process of viral infection, such as binding to receptors (Daniels, 1975), although occasionally they are directed against neuraminidases (Webster et al., 1982; Laver et al., 1982). In the enveloped viruses, the surface glycoproteins are responsible for the early events in cellular infection, and neutralizing antibodies are directed against these proteins. In a well studied system, such as the orthomyxoviridae, a number of antigenic sites can be defined on the hemagglutinin, which is the glycoprotein responsible for binding to cellular receptors. Each antigenic site consists of a cluster of epitopes, the combining site of a single monoclonal antibody, and by definition, alteration of the epitopes on one site does not affect the epitopes on another site (Gerhard et al., 1981).

La Crosse (LAC) virus, a member of the California serogroup of the family Bunyaviridae, contains two glycoproteins in its envelope, G1 (125kD) and G2 (34kD) (Obijeski et al., 1976). Three laboratories have now established that monoclonal antibodies directed against G1 may have high neutralizing activity (Gonzalez-Scarano et al., 1982; Kingsford et al., 1983, Grady et al., 1983a), and are frequently capable of inhibiting hemagglutination (Gonzalez-Scarano et al., 1982). The large glycoprotein therefore appears to have receptor binding and hemagglutinating activity. It is also

involved in cell-to-cell fusion, since it undergoes a conformational change at the pH that will activate the fusing activity of LAC virus (Gonzalez-Scarano et al., 1984; Gonzalez-Scarano, 1985). Furthermore, most neutralizing antibodies also block fusion from within (Gonzalez-Scarano et al., unpublished, 1984). Previous studies (Gonzalez-Scarano et al., 1982; 1983) have established that in addition to neutralizing antibodies against the G1 protein, there is a group of non-neutralizing anti-G1 monoclonal antibodies; most non-neutralizing antibodies also fail to inhibit hemagglutination or fusion (Gonzalez-Scarano and Pobjecky, unpublished, 1984). Among both groups, some antibodies are type-specific while others cross-react with several California serogroup viruses.

Two major techniques have been used to define antigenic sites with monoclonal antibodies: i) competitive binding assays and, ii) selection of variant viruses (Gerhard et al., 1981; Wiktor and Koprowski, 1980; Lafon et al., 1983; Parrish and Carmichel, 1983). Each method has its disadvantages. Competitive binding assays can underestimate the number of epitopes, since competition for binding sites by two antibodies may be due to steric hindrance rather than to identity of the combining site (Lubeck and Gerhard, 1981). Selection of variant viruses, on the other hand, is limited to strongly neutralizing antibodies (Wiktor and Koprowski, 1980; Lefrancois and Lyles, 1983; Birrer et al., 1981). Competition assays have been used previously (Grady et al., 1983b; Kingsford et al., 1983) to study the relationship between epitopes on the G1 protein of La Crosse virus.

We have selected a group of antigenic variants of LAC virus and studied them by neutralization tests and by binding in an ELISA. The results show that the epitopes on the G1 protein form clusters or regions which appear to be part of a large antigenic site. The data from the binding assays correlate very well with the results obtained with neutralization tests and support the delineation of distinct but related epitope groups.

## METHODS

Monoclonal antibodies. The selection and cloning of hybridomas secreting antibodies against LAC virus proteins has been described previously (Gonzalez-Scarano, et al., 1982). Other monoclonal antibodies not included in the original report were obtained by using a different immunization protocol. Briefly, about 1 mg of LAC was suspended in 1 ml of 25mM sodium acetate, pH 5.3, and held for 30 min at 4° then neutralized with 1M Tris-HCl, pH 8.0, and UV-irradiated for 20 min at room temperature using the ultraviolet light of a biosafety hood. Six-week-old BALB/c mice (Charles River Laboratories, Portage, Michigan) were then inoculated intraperitoneally with 150-250 ug of treated LAC homogenized with an equal volume of complete Freund's adjuvant. Four weeks later mice were re-immunized with 50-100 ug of acidified LAC and the spleens harvested for fusion 2-4 days later. The construction of hybridomas and their screening for antibody secretion followed our published protocol (Gonzalez-Scarano et al., 1982).

Selection of Variant Viruses. Ten-fold dilutions of LAC virus original strain, in the form of a tissue culture stock or a 10% suckling mouse brain homogenate were combined with a low dilution (1:5 or 1:10) of a mouse ascitic fluid containing a monoclonal antibody and held for 30 min at room temperature or overnight at 4°. The virus was then inoculated on a monolayer of BHK-21 (clone 13) cells on 6-well plates and rocked gently for 60 min at room temperature, following which the inoculum was removed and the monolayer overlayed with Eagles Modified MEM (EaMEM) containing 2% fetal calf serum (FCS) and a final concentration of 0.5% agarose (Seaplaque, Marine Colloids Division, Rockland, Maine). Isolated plaques appearing at the end-point of neutralization were picked and replaques twice, again combining the virus with a low dilution of the appropriate ascitic fluid prior to inoculation on a BHK -21 monolayer. The frequency of selection of variants from each monoclonal antibody was calculated as (Titer of LAC Stock + Ascitic Fluid)/Titer of LAC Stock. Tissue culture stocks were



then prepared in the presence of a 1:25 dilution of ascitic fluid containing the monoclonal antibody.

Neutralization Tests. BHK-21 cells were seeded on 96-well plates and maintained with EaMEM supplemented with 10% FCS. When the cells were confluent, the growth medium was removed and the monolayer was inoculated with two-fold dilutions of monoclonal ascitic fluid diluted in EaMEM with 2% FCS. One hundred plaque forming units of either LAC or variant virus were then added to each well and the plates were incubated at 35°. On the third day after inoculation, the monolayers were scored for the presence of cytopathic effect. The neutralizing titer of each antibody was the highest dilution that prevented 50% CPE in more than half of the wells.

Neutralizing monoclonal antibodies had titers in the range of 1:320 to 1:5120, except for antibody 807-09 which had a titer of 1:100 and antibody 807-31 which had a titer of 1:100,000. Tests with variant viruses were scored as neutralization if titers were no more than 2-fold lower than the titer against parent LAC virus. No neutralization was defined as the inability of an antibody to neutralize a variant virus at a dilution of 1:40, in spite of that antibody's ability to neutralize parent LAC virus at that concentration. However, in most cases the failure to neutralize was absolute. Because antibody 807-09 had a low neutralizing titer against parent LAC, its neutralization of two variants at a dilution of 1:20 was listed as partial neutralization. Antibody 807-25 had been reported as non-neutralizing (Gonzalez-Scarano et al., 1982) but was subsequently discovered to have neutralizing activity by testing different harvests of ascites fluid.

ELISA. LAC and variant viruses were grown on BHK-21 cells and purified by precipitation with polyethylene glycol (PEG 8000, Fisher Scientific) and banding on sucrose gradients as described previously (Gonzalez-Scarano, 1985). ELISA assays were carried out as described (Gonzalez-Scarano, 1985), with some modifications. Protein concentrations were measured by absorbance at 280 nm, after determination of the attenuation coefficient using the Bio-Rad assay. Because of the importance of having equal amounts of antigen prepared with each variant virus in the ELISA, the antigens were further calibrated on polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970). Equivalent amounts of protein were loaded on a 5.0% polyacrylamide stacking gel after boiling for 3 min in Laemmli's Sample Buffer (Laemmli, 1970), and resolved in 12.5% gels. The gels were then stained with silver (Switzer et al., 1979) and the nucleocapsid band was scanned with an LKB Zeinek densitometer. The densitometer scans were weighed on an analytical balance and minor adjustments then made of the concentration of all variant antigens. We had previously determined, using LAC virus, that, over the range of concentrations tested, the degree of nucleocapsid protein staining with silver is proportional to the amount of protein loaded on the gels (data not shown). Approximately 250 ng were placed on each well of microelisa plates (MicroElisa, Cooke), and held overnight at 4°. Assays followed a published protocol (Gonzalez-Scarano, 1985). In at least two separate experiments, each antibody was tested in quadruplicate at two dilutions, and the results quantified as ((binding to variant/binding to LAC) x 100) after subtraction of background (binding to either variant virus or to LAC virus by ascites fluid obtained by injection of parent myeloma cells into BALB/c mice).

## RESULTS

Variant viruses. A panel of 11 neutralizing monoclonal antibodies against the G1 protein were used to select variant viruses. The monoclonal variants occurred at frequencies ranging from  $10^{-4.0}$  to  $10^{-6.4}$  (Gonzalez-Scarano et al., 1983). The frequency of selection of variants was similar to that reported for other RNA viruses (Lubeck et al., 1980; Porter et al., 1980). Approximately 110 variants were selected with the panel of neutralizing antibodies and most were used in neutralization assays while only prototype viruses were tested by ELISA. All of the variants grew in BHK-21 cells, but not to the same extent, necessitating some adjustment of the antigens prepared for ELISA (see Methods). Variants selected with antibody 807-22 have been studied extensively (Gonzalez-Scarano et al., 1985) because they demonstrated reduced neuroinvasiveness, reduced ability to mediate cell-to-cell fusion, and an alteration of the pH required to activate fusion.

Neutralization assays. Cross-neutralization assays with each of the 11 monoclonal antibodies against one variant per epitope are shown in Fig. 1. The results were scored as neutralization, partial neutralization, or no neutralization, as defined in Methods. The neutralizing antibodies fell into five distinct groups, with three of the groups represented by a single antibody. The largest cluster, headed by antibody 807-31, was mainly composed of antibodies that are strain specific, and it represented the major neutralizing region of G1. Antibodies 807-12 and 807-33 appeared identical by this analysis, but in fact showed differences when a panel of California viruses was used in neutralization assays with them (Gonzalez-Scarano et al., 1983).

Approximately 100 additional antigenic variants selected with the same monoclonal antibodies were tested in cross-neutralization assays. Although there were

variations in the degree of neutralization obtained with different variants selected with a single monoclonal antibody, all variants selected with the same monoclonal antibody had the same pattern of cross-neutralization (data not shown).

ELISA. The 11 variant viruses (one variant per epitope) were tested against the 11 neutralizing monoclonal antibodies and 8 non-neutralizing monoclonal antibodies directed against the G1 protein. Binding of each monoclonal antibody to parent LAC virus was defined as 100% and binding to variants was scored relative to this standard, after equalizing the amount of each variant used (see Methods). Binding to variants ranged from 0% to 100% (or occasionally more than 100%), providing dramatic differences and the clear cut patterns shown in Fig. 2.

Almost all of the monoclonal antibodies showed significantly reduced binding by ELISA to the variant viruses selected with them; for most of the antibodies there was, in fact, no binding above background (Fig. 2). Antibodies 807-9 and 807-22 bound the variants selected with them when tested in ELISA, at levels of 100% and 69% respectively, in spite of their inability to neutralize these variants.

Many of the monoclonal antibodies also demonstrated reduced or no binding to variants selected with other antibodies, producing a pattern of interrelationships. The results obtained with ELISA are in excellent overall agreement with the findings of the neutralization tests (Fig. 1). There are five groups that include at least one neutralizing antibody. The largest cluster, again headed by antibody 807-31, now consists of 9 antibodies, since three non-neutralizing antibodies map to this area. The group headed by antibody 807-22 now also includes three non-neutralizing antibodies that show diminished binding to variant 22. These four antibodies also demonstrate reduced binding to acid-treated virus (Gonzalez-Scarano, 1985). We had previously proposed that they comprise a cluster of epitopes which is affected by the conformational change that G1 undergoes at the pH of fusion.

Variant 25, which had appeared to be a separate isolated epitope in cross-neutralization assays, now appears closely related to variant 22, since it shows reduced binding of the four antibodies defining epitope 22. In addition, epitope 25 now is related to two other groups of antibodies (those headed by 807-31 and 807-12), as well as to two non-neutralizing antibodies (807-21 and 807-26) which seem to bear little relationship to the other four epitope groupings.

Comparison of neutralization and ELISA results. A comparison of the binding and cross-neutralization data is presented in Table I. Over 85% (23/27) of the antibody-virus combinations that showed no neutralization also showed decreased binding in ELISA (0 - 74%). Similarly, 95% (88/92) of the antibody-virus combinations that showed neutralization showed binding on ELISA that was close to control values (75-100+%).

There were four instances where neutralizing antibodies failed to neutralize variants to which they bound strongly (75%-100%), i.e., antibody 807-09 (variant 9), antibody 807-18 (variants 31 and 35), and antibody 807-15 (variant 13). There are two instances where antibodies that neutralized a variant did not bind it on ELISA (variant 18 and antibodies 807-9 and 807-15). In this latter instance, both antibodies immunoprecipitated the G1 protein of the variant virus. In addition, by altering the ELISA conditions - using phosphate buffered saline instead of sodium bicarbonate in the binding buffer - one of the antibodies (807-9) bound to variant 18 as well as parent LAC.

## DISCUSSION

This analysis of the antigenic structure of the G1 glycoprotein of LAC virus leads to two major conclusions. On the one hand, the epitopes identified by monoclonal antibodies can be separated into distinct groups or clusters. The neutralization assays define 5 such clusters. On the other hand, the data suggest that these 5 epitope clusters may be part of a single immunodominant antigenic site, since the ELISA results indicate interrelationships between all of the groups, with the exception of the single monoclonal antibody 820-260. Of particular importance, the data do not suggest that there is a separate non-neutralizing antigenic site as previously postulated (Gonzalez-Scarano et al., 1982; Kingsford and Hill, 1983).

This study confirms the importance of using several assays in an epitope analysis, since each method can provide additional information. We used the neutralization test because it is probably the best indicator of antigenic differences and because it is biologically highly relevant. The ELISA assay complements neutralization, since it measures a different parameter of virus-antibody interaction and since it is sensitive to interrelationships between epitopes.

There was an excellent correlation between the results of the ELISA and the cross neutralization studies, suggesting that most of the monoclonal variants escaped neutralization through diminished binding. However, there were a few striking discrepancies. In a few instances (4 of 121), antibodies bound strongly but failed to neutralize (Table 1). For instance, antibody 807-09 bound strongly to variant 9, but failed to neutralize it (Figs. 1 and 2). A similar phenomenon is illustrated by the 8 non-neutralizing antibodies, all of which apparently bound to neutralizing epitopes. Binding of monoclonal antibodies to variants without neutralization has been reported for other

viruses (Roehrig et al., 1985), and there may be several explanations, such as low antibody-antigen affinity or multiple steps in the antibody-virus interaction leading to neutralization.

More paradoxical is the failure, in 2 of 121 instances (both with variant 18), of an antibody to bind even though it neutralizes effectively (Table I). It seems likely that the procedure used to coat virus on to ELISA plates, including alkaline pH, altered the conformation of epitope 18, since both of the antibodies immunoprecipitated the G1 protein of variant 18.

Relationships between epitopes on the G1 protein of LAC virus have been studied by two investigators (Kingsford et al., 1983; Grady et al., 1983b) using competition assays. Both of these studies identified clusters of closely related epitopes. If a limited number of antibodies were used (Grady et al., 1983b), the epitope clusters appeared totally unrelated. However, where a large panel of antibodies were included (Kingsford et al., 1983), an interlinked pattern of epitopes appeared, consistent with a single immunodominant antigenic site. Furthermore, both neutralizing and non or weakly neutralizing antibodies fell into a single continuum; there was no evidence of a topographically distinct non-neutralizing antigenic site. Thus, it appears that there is good congruence between the present study, utilizing the variant virus approach, and prior work utilizing competition assays.

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TABLE I

Neutralizing anti-GI protein monoclonal antibodies and the frequency and virulence of antigenic variants selected by them

Epitope Group	Antibody Number	Immunizing Virus	Specific(S) or Cross-Reactive (C)	Frequency of Variants (log 10)	Virulence
1	807-31	LAC	S	-6.0	+
	807-09	LAC	S	-3.7	+
	807-35	LAC	S	-5.3	+
	807-13	TAH	C	-5.9	+
	807-18	LAC	S	-5.3	+
	807-15	LAC	S	-6.2	+
2	820-260	LAC	ND	-5.4	+
3	807-12	LAC	C	-5.0	+
	807-33	LAC	C	-4.1	R
4	807-25	LAC	ND	-6.4	+
5	807-22	LAC	C	-6.3	R

\* Characteristics of these antibodies have been published (Gonzalez-Scarano et al., 1982; 1983). Specificity was determined in neutralization tests with a panel of California serogroup viruses. Selection of variants is described in the methods section. Virulence was based on subcutaneous injection of suckling mice with 1000 pfu (+: as virulent as parent virus; R: reduced by comparison with parent virus).

TABLE 2

A comparison of neutralization and binding (ELISA) tests on 11 neutralizing monoclonal antibodies and the 11 variant viruses selected by them\*

Neutralization	ELISA			Totals
	0%	1-74%	75-100%	
None	14	9	4	27
Partial	0	1	1	2
Complete	2	2	88	92
Totals	16	12	93	121

\* Based on data in Figs. 1 and 2.

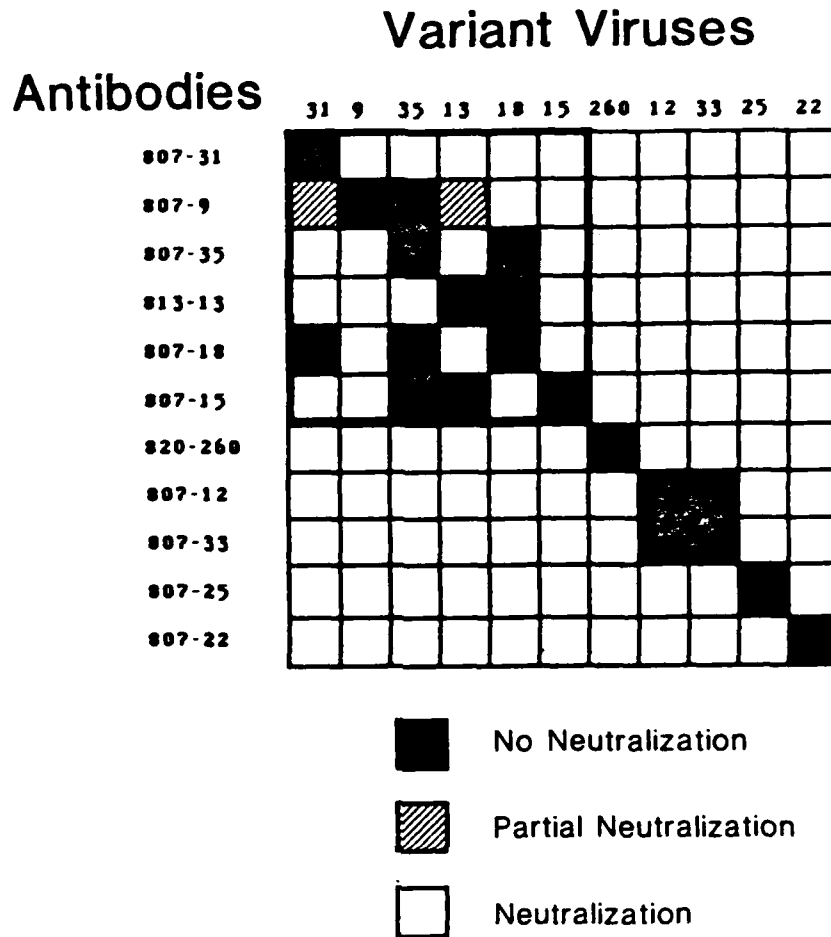


Fig. 1. Neutralization of antigenic variants by the monoclonal antibodies used to select them. The 11 antibodies shown neutralized parent La Crosse virus at titers ranging from 1:320 to 1:100,000. No neutralization was defined as the inability of an antibody to neutralize at a dilution higher than 1:20. Neutralization was defined as a titer against a variant virus which was no less than 2-fold below the titer of the same antibody against parent La Crosse virus. Partial neutralization was seen only for antibody 807-09, as described in Methods.

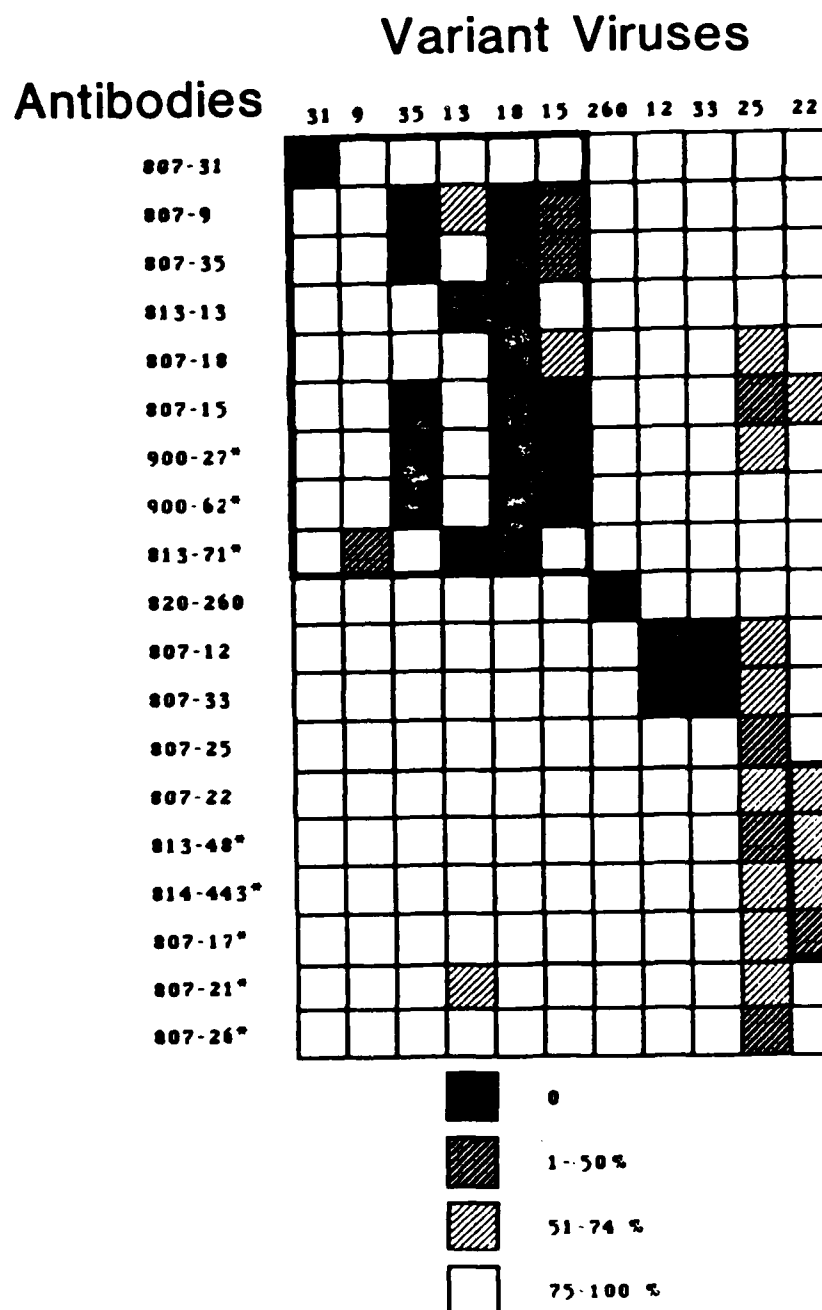


Fig. 2. Binding of anti-G1 glycoprotein monoclonal antibodies to antigenic variants in ELISA. Binding to variant viruses is recorded relative to binding of the same antibody to parent La Crosse virus. Values greater than 100% were grouped in the 75-100% category. Binding in the 1-50% category was almost always in the 20-50% range, with two exceptions. Asterisk (\*) indicates non-neutralizing antibodies. The two boxes define one large epitope group and an acid-sensitive epitope group, respectively.



Appendix 6

Draft II

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Polygenic determination of the virulence of reassortants between  
La Crosse and Tahyna viruses

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NOTE: This is a report of progress and not a complete manuscript.

## ABSTRACT

A virulent strain of La Crosse (LAC/original) virus and an avirulent strain of Tahyna (TAH/181-57) virus were used to construct reassortants. In order to avoid multiple silent mutations, wild-type viruses were used. For rapid phenotyping of reassortants, plaques from doubly infected BHK-21 cell cultures were typed by SDS-PAGE, exploiting differences in the migration of the G1 glycoprotein and of the nucleocapsid (N) protein of the two viruses. Tentative phenotypes were confirmed by ELISA with type-specific monoclonal antibodies. To obtain a complete genotype on putative reassortants, RNA-RNA hybridization was used.

A total of 291 clones from doubly infected cultures were phenotyped and 30 selected clones were then genotyped. A total of 13 clones, representing all possible reassortant genotypes, were obtained. These clones were then titrated by subcutaneous injection of suckling mice and an pfu/LD50 ratio computed. Incomplete testing in mice has yielded several conclusions: (1) Reassortants with a virulent M RNA segment resemble parent La Crosse virus in virulence. (2) Reassortants with an avirulent M RNA segment are more virulent than the avirulent parent Tahyna/181-57 virus. (3) The M RNA segment is the most important genetic determinant of virulence, but the virulence phenotype may be modulated by the other two viral genes (L RNA and S RNA segments), for certain genetic reassortants.

## INTRODUCTION

We have previously described and compared the pathogenesis of virulent LAC/original and avirulent THA/181-57 viruses (Janssen et al, 1984). To identify the gene or genes which determine virulence, we wished to construct reassortants between these two parental viruses, avoiding the use of ts mutants because of the problem of silent (non-ts) mutations which could alter the biological properties of the reassortants (Rohzon et al, 1981). Two methods for phenotyping were used, SDS-PAGE of the viral proteins and ELISA with monoclonal antibodies. These permitted the rapid phenotyping of large numbers of plaques from dually infected cultures, but failed to type the product of the third viral gene, the L RNA segment. RNA-RNA hybridization was then used to genotype all 3 RNA segments of selected putative reassortants.

## METHODS AND RESULTS

### Production of reassortants

BHK-21 monolayers on 24-well plates (COSTAR) were co-infected with LAC/original and TAH/181-57 viruses at several multiplicities. After 30 minutes, the inoculum was removed and the plates incubated at 33°C degrees for 18-24 hours. Each well was harvested, stored in 20% FCS at -80°C, and later titrated in a standard plaque assay. Individual plaques were grown on BHK-21 monolayers to make mini-pools.

### Phenotyping by polyacrylamide gel electrophoresis

A single well in a 24 well plate containing a BHK monolayer, was infected at an MOI of 1.0, incubated 16 hours, pulse labelled for 1.5 hours with 35S methionine, and lysed with Laemmli's sample buffer (Laemmli, 1970). The cell lysate was run on reducing SDS-PAGE and fluorographed. As shown in Figure 1, the G1 protein of LAC/original virus migrates more rapidly, while the nucleocapsid (N) protein of LAC/original migrates more slowly, than the corresponding proteins of TAH/1891-57.

### Phenotyping by ELISA with monoclonal antibodies

An alternative approach to rapid phenotyping is the use of monoclonal antibodies against the G1 and N proteins of LAC and TAH viruses. By selecting antibodies which are relatively specific for each virus it is possible to do a rapid "pattern" test. The method for preparing ELISA antigens and conducting the test has been published (Gonzalez-Scarano et al, 1982). Table I shows that when ELISA is applied to seven selected reassortants the patterns confirm the tentative conclusions from PAGE.

### Genotyping by RNA-RNA hybridization

To complete the assignment of genotype and to confirm typing of the products of the M RNA and S RNA segments, we resorted to direct genotyping. The method introduced by Hay (Hay et al, 1977, 1979) for influenza virus was modified in a number of ways.

BHK-21 cells were inoculated with an MOI of 0.01 and incubated in phosphate-free MEM for two hours. P32 orthophosphate was then added, 2 mCi per T175 flask, and cultures were incubated for 44 hours post infection. Virus was precipitated from the supernate and banded on a sucrose gradient. Virus RNA (vRNA) extracted from banded virus served as the labelled (-) stranded partner in hybridization reactions.

Virion complementary (vc) RNA provided the unlabelled (+) strand partner in hybridization. BHK-21 cells in 10cm petri dishes were infected with an MOI of 50 and cultures were harvested after 6 hours of infection. Cells were washed and lysed with TSM buffer containing 0.25% NP-40. The suspension was centrifuged for 14 minutes at 2800 RPM to sediment nuclei and the supernatant was treated with 0.2% SDS, 7M urea in tris buffer. The suspension was then twice extracted with phenol-chloroform, followed by chloroform:isoamyl alcohol, and the RNA was precipitated with cold absolute ethanol.

For hybridization, 90000 DPM of vRNA and vcRNA harvested from 3/5 of a petri dish were combined. RNAs were brought up in 90% DMSO, denatured at 45°C for 30 minutes, and renatured at 37°C for 16 hours. The RNA was ethanol precipitated, and digested with S1 endonuclease, to degrade single stranded molecules. RNA was phenol extracted, ethanol precipitated, and electrophoresed as denatured RNA strands in formaldehyde buffered 1% agarose gel. Dried gels were autoradiographed for 8-16 hours.

Figure 2 presents an example of results. La Crosse virus vRNA is well protected against S1 digestion with La Crosse vcRNA but not with Tahyna vcRNA. vcRNA preparations from 3 reassortant viruses give striking differential protection to La Crosse vRNA, permitting unambiguous genotyping.

#### Construction of reassortant viruses

A series of co-infections of BHK-21 cells with La Crosse (LAC) and Tahyna (TAH) viruses were carried out, and 291 individual clones were phenotyped by protein electrophoregrams (Table 2). These crosses yielded two sets of reassortants, 5 with phenotype XTL and 31 with phenotype XLT. RNA-RNA hybridization was used to further type 10 of these reassortants, yielding the 4 genotypes LLT, TLT, TTL, and LTL (Table 3). A limited number of reassortants with XTT and XLL were genotyped by RNA-RNA hybridization; from 15 tested, one reassortant (LTT) was identified. To obtain reassortants of genotype TLL, a new cross was made between reassortants F1-2a (TTL) and B1-11a (TLT); 28 clones were phenotyped and two with XLL proteins were identified. These two reassortants were then shown to be genotype TLL by hybridization.

A panel of reassortants, shown in Table 3, was then assembled. A total of 13 reassortants was composed of 1-3 representatives of each of the 6 possible reassortants.

#### Virulence of reassortants

To obtain a quantitative assessment of the 13 reassortants, each virus was titrated by the subcutaneous (sc) route in suckling CD-1 mice; the pfu titer was divided by the sc LD50 titer to yield an index (pfu per LD50) of virulence.

Incomplete results are summarized in Table 3. Those reassortants with a La Crosse M RNA segment show a virulence similar to that of parent La Crosse virus. There is one exception, clone B1-10a, which may be a spontaneous mutant since it has a small plaque phenotype, not seen in either parent virus.

Reassortants with a Tahyna M RNA segment show considerably greater virulence than parent Tahyna virus, and can be classified as intermediate between the two parental strains. Data are not sufficiently complete to know whether the L RNA as well as the S RNA segments (from La Crosse virus) can modulate the effect of the Tahyna M RNA segment.

## DISCUSSION

Shope and Bishop (1981) reported that, within the California serogroup, neuroinvasiveness co-segregates with the M RNA segment. The present work represents an advance over these earlier studies in that we have used wild-type viruses rather than ts mutants for the construction of reassortant viruses. It has been shown (Rozhon et al, 1981) that ts mutants may carry silent mutations which alter their virulence character.

A second advantage of the present study is the selection of an avirulent prototype virus (Tahyna 181-57) which is much less virulent than the strain (Tahyna B92) used previously (Shope and Bishop, 1981). This makes it possible to identify strains of intermediate virulence and to look for polygenic effects. In addition, the avirulent Tahyna 181-57 strain has helped to clarify differences in pathogenesis.

The major new finding which emerges from the present study is the polygenic determination of virulence. It appears that, with reassortants possessing the avirulent M RNA, the presence of one or both of the other segments from La Crosse virus confers an intermediate virulence which is distinctly greater than that of the avirulent Tahyna 181-57 parent. Apparently, this modulating effect can be conferred by the S RNA and perhaps also by the L RNA segment. The assay system is not sufficiently refined to show whether there is an additive effect of these two genes.

In sum, these studies indicate that the M RNA segment plays a dominant role in the determination of virulence. The middle RNA segment of bunyaviruses encodes the two envelope glycoproteins G1 and G2 (Bishop and Shope, 1979). These proteins mediate the first two steps in infection, that is, attachment to cellular receptors and fusion between the viral envelope and a host membrane (Gonzalez-Scarano et al, 1982, 1984).



Alterations in either of these functions could influence replication in key target cells such as myocytes and thereby regulate virulence. Independent evidence that the G1 protein does indeed play a role in virulence comes from recent work (Gonzalez-Scarano et al, 1985) showing that selected variant viruses, representing mutants in the G1 protein, have reduced virulence.

The mechanism where by the other RNA segments could modulate the effect of the M RNA segment is entirely unknown. A muscle culture system, capable of providing a model for the differential myotropism of La Crosse and Tahyna viruses, might help to elucidate these polygenic effects.

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Table 1

Typing of prototype and reassortant viruses with monoclonal antibodies

Virus	Monoclonone*		C	D	G1		N		Genotype
	A	B			Pattern	Protein	Pattern	Protein	
<u>Prototypes</u>									
LLL	100K	12K	400	800	Hi/Lo	LAC	Lo/Lo	LAC	XLL
LTL	25K	100K	1600	1600	Lo/Hi	TAH	Lo/Lo	LAC	XTL
TTT	6K	200K	1600	6K	Lo/Hi	TAH	Lo/Hi	TAH	XTT
TLT	200K	3K	200	800	Hi/Lo	LAC	Lo/Hi	TAH	XLT
<u>Reassortants</u>									
AI-3a	200K	50K	100	400	Hi/Lo	LAC	Lo/Hi	TAH	XLT
BI-10a	200K	25K	200	1600	Hi/Lo	LAC	Lo/Hi	TAH	XLT
BI-11a	200K	50K	800	1600	Hi/Lo	LAC	Lo/Hi	TAH	XLT
BI-26a	200K	25K	400	3200	Hi/Lo	LAC	Lo/Hi	TAH	XLT
BI-29a	200K	100K	400	800	Hi/Lo	LAC	Lo/Hi	TAH	XLT
FI-2a	200	200K	400	400	Lo/Hi	TAH	Lo/Lo	LAC	XTL
FI-18a	200	200K	400	400	Lo/Hi	TAH	Lo/Lo	LAC	XTL

\* A: anti-G1 (LAC) 807-35; B: anti-G1 (TAH) 813-77; C: anti-N (LAC) 807-28; D: anti-N (TAH) 814-02. Note that ELISA phenotyping agrees with the results from PAGE.

Table 2

Summary of reassortants generated by co-infections  
between LAC/original and TAH/181-57 viruses

Experiment Number	MOI LAC/TAH	Total	Number of Reassortants				
			XLL	XTT	XXX	XTL	XLT
A1 0.5/0.5	26	18	0	6	0	2	
B1 0.5/2.0	28	16	7	1	0	4	
E1 25/25	6	5	0	1	0	0	
F1 5/5	20	3	10	2	2	3	
F2 5/5	28	1	12	8	2	5	
F3 5/5*	14	0	11	0	0	3	
F4 5/5*	21	1	18	0	1	1	
G1 5/10	16	4	11	0	0	1	
G2 5/10	24	3	18	1	0	2	
G3 5/10*	20	4	12	2	0	2	
G4 5/10*	24	0	20	0	0	4	
H1 5/25	4	0	4	0	0	0	
H2 5/20	27	0	18	4	0	5	
H3 5/25*	12	0	10	2	0	0	
H4 5/20*	21	0	21	0	0	0	
Totals		291	55	172	17	5	31

\* Monoclonal antibody 807-31 (0.1 ml of 1:1000 dilution) against LAC/original virus (type-specific) was added to these infections. These clones were phenotyped by polyacrylamide gel electrophoresis. MOI: multiplicity of infection.

Table 3

Mortality and survival following subcutaneous infection of suckling mice with parental LAC/original and TAH/181-57 viruses or of reassortant clones

Virus Clone	Phenotype	Genotype	PFU/LD50 sc injection	Virulence
LAC/ori	XLL	LLL	14	+
TAH/181	XTT	TTT	120,000	-
A1-3a	XLT	LLT	8	+
B1-29a	XLT	LLT	14	+
B1-11a	XLT	TLT	2	+
B1-26a	XLT	TLT	2	+
B1-10a*	XLT	TLT	711*	+/-
P1-26b <sup>+</sup>	XLL	TLL	?	
P1-13c <sup>+</sup>	XLL	TLL	?	
F1-2a	XTL	TTL	1473	+/-
F2-2a	XTL	TTL	?	?
F1-18a	XTL	LTL	245	+/-
F2-18a	XTL	LTL	7535	+/-
F4-5a	XTL	LTL	?	?
B1-1a	XTT	LTT	?	?

\* Possibly a spontaneous mutant, since it produced small plaques as well as prolonged survival of mice.

+ (Obtained from a cross of TTL and TLT).

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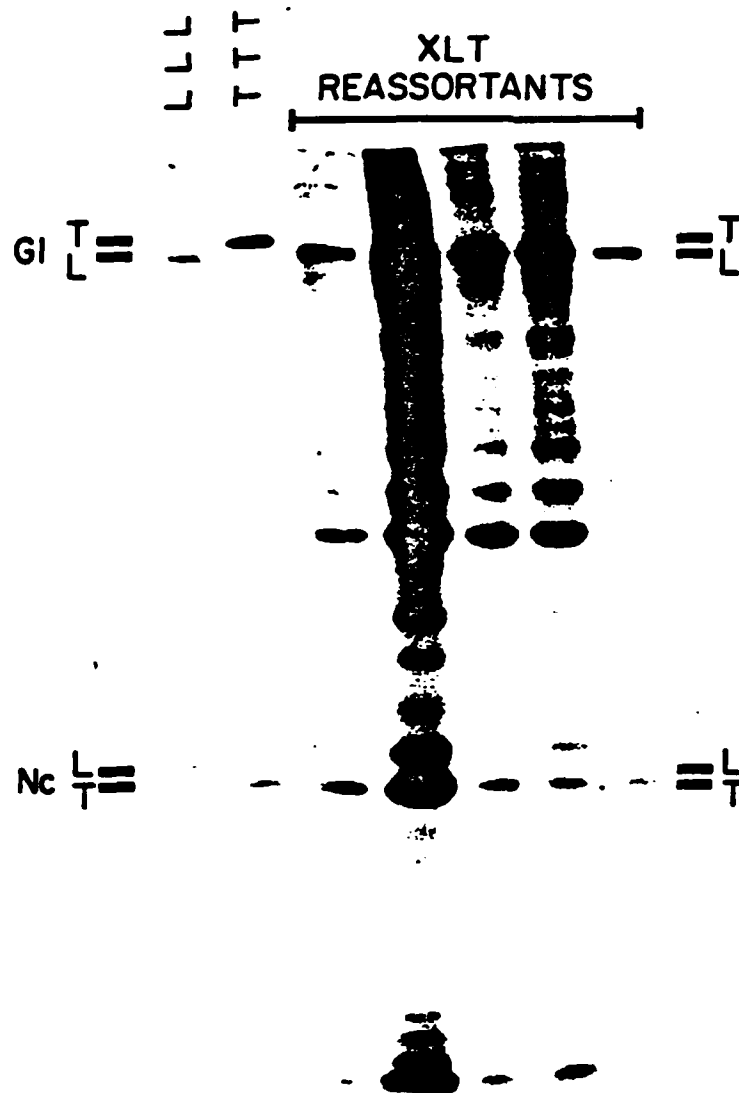


Figure 1. Autoradiograph of a 10% polyacrylamide gel showing the GI and N proteins of parent LAC/original and TAH/181-57 viruses and of 5 reassortants, all with genotype XLT. Note that the GI (and G2, poorly labelled) of LAC migrates faster, and the N of LAC migrates more slowly, than the corresponding proteins of TAH virus.

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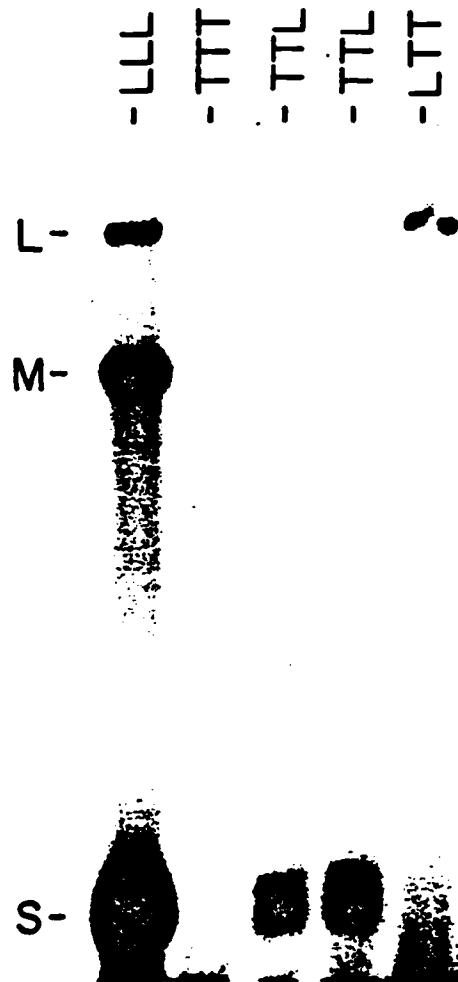


Figure 2.

RNA-RNA hybridization for the genotyping of reassortant viruses. P32-labelled virion RNA (vRNA) from La Crosse virus has been hybridized with the following preparations of cold cellular virion-complementary RNA (vcRNA) from virus-infected BHK cultures. Lane 1: La Crosse cRNA; Lane 2: Tahyna cRNA; Lane 3: reassortant of genotype TTL; Lane 4: reassortant of genotype TTL; Lane 5: reassortant of genotype LTT. Note that the S RNA band always migrates as a dimer, because (+) strand vcRNA occurs as both a full length transcript and as a subgenomic transcript.



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